Mémoire

présenté à

l'Université Blaise Pascal

(Clermont-Ferrand II)

pour obtenir

l'Habilitation à Diriger des Recherches

par

Olivier MONTEUUIS

Soutenu publiquement le 1er Octobre 1999 devant la Commission d'Examen composée de:

M. Georges DUCREUX,	Rapporteur
M. Jean-Michel FAVRE,	Rapporteur
M. Michel GENDRAUD,	Rapporteur
M. Daniel CORNU,	Examinateur
M. Claude EDELIN,	Examinateur
M. André FRANCLET,	Examinateur
M. Gilles PETEL,	Examinateur

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M. André FRANCLET,	Examinateur
M. Gilles PETEL,	Examinateur

Je tiens à remercier l'ensemble des membres du Jury, et plus particulièrement MM. les Rapporteurs, pour avoir accepté de juger ce travail.

Je sais gré à l'Association Forêt Cellulose (A.FO.CEL), l'Université Blaise PASCAL, l'Université du Minnesota, le CIRAD-Forêt et Innoprise Corporation Sdn Bhd pour m'avoir fait bénéficier des conditions d'environnement qui ont contribué à l'accomplissement de ce travail.

SOMMAIRE GÉNÉRAL

I. CURRICULUM VITAE

Nom	MONTEUUIS
Prénom:	Olivier
Date de naissance:	2 Juin 1957
Nationalité:	Française
Situation familiale:	Marié, deux enfants nés en 1990 et 1993
Adresse actuelle:	CIRAD-Forêt Campus de Baillarguet BP 5035 34032 MONTPELLIER Cedex 1 FRANCE
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LANGUES ETRANGERES: Anglais, Allemand.

FORMATION:

- **Ingénieur horticole**
  Diplômé en 1980 de l'Ecole Nationale des Ingénieurs des Techniques Horticoles et du Paysage (ENITHP) d'Angers.

- **D.E.A. de Biologie et Physiologie végétales**
  Diplômé en 1984 de l'Université Blaise Pascal de Clermont-Ferrand;

Thème de Recherche: Etude de la multiplication végétative de *Sequoiadendron giganteum* en vue du clonage.

- **Doctorat d'Etablissement en Biologie et Physiologie végétales**
Diplômé en 1988 de l'Université Blaise Pascal de Clermont-Ferrand;

Sujet de Thèse: Aspects du clonage de séquoias géants (*Sequoiadendron giganteum*) jeunes et âgés: Culture in vitro d'extrémités végétatives apicales; Comparaison des deux types de matériels.

STAGES:

- Serres et pépinières privées:

- 1978 - 1979 : * Ets Richard (France)
* Pépinières Agrapart (France)
* Taille de vergers palissés (France)

- 1979 : * Pépinières Delbard International (France)

- 1982 : * Pépinières Euro-Baumschule R. Schmidt (Allemagne)

- Centre de Recherche:

- 1980 : * Institut National de la Recherche Agronomique (INRA)

Sujet d'Etude: Applications de la culture in vitro à l'amélioration des arbustes ornementaux:
. Optimisation des milieux de culture in vitro
. Traitements mutagènes

EXPERIENCES PROFESSIONNELLES:

- **1982 - 1987** : * **Responsable de la pépinière expérimentale et de production de l'Association Forêt-Cellulose** (AFOCEL) à Marvejols (Lozère).

*** Responsable du Programme AFOCEL "Résineux divers"** incluant:

. l'Amélioration génétique

. les Méthodes culturales

- 1988 - Février. 1991 : * Directeur Adjoint de la station de Biotechnologies Forestières de l'AFOCEL (Etançon, France), spécialisé dans les techniques de rajeunissement en vue du clonage conforme de génotypes sélectionnés âgés, et dans la production de plants forestiers:

- en laboratoire de culture in vitro:
. Culture de méristèmes
. Microgreffage de méristèmes
. Micropropagation
. Rhizogenèse
. Acclimatation des vitroplants produits

- en pépinière d'expérimentation et de production:
. Gestion et conduite technique de serres et de pépinières modernes (plants hors-sols);
. Conditionnement physiologique des pieds-mères;
. Propagation par graines, greffage, marcottage et bouturage;
. Acclimatation, sevrage et élevage de plants issus de culture in vitro et de techniques horticoles plus classiques.

- Mars 1991 - Avril 1997: * Directeur du Laboratoire commun ICSB/CIRAD-Forêt de Biotechnologies Forestières de Tawau (Sabah, Malaisie orientale, Ile de Bornéo).

Principales réalisations: Mise au point de protocoles adaptés à la culture in vitro de:
Calamus manan, *C. subinermis* et *C. merrillii* (Rotins), incluant l'embryogenèse somatique;
Tectona grandis (teck): production clonale industrielle à partir de génotypes sélectionnés âgés;
Acacia mangium, *A. auriculiformis* et hybrides interspécifiques;
Paraserianthes falcataria.

* **Appui au projet commun ICSB/CIRAD-Forêt "Production de plants améliorés" au Sabah** (Malaisie orientale, île de Bornéo).

Principales réalisations: Mise au point de techniques pour la production de matériels génétiquement améliorés pour:
Tectona grandis (teck): multiplication clonale industrielle par bouturage horticole de génotypes sélectionnés âgés;
Acacia mangium, *A. auriculiformis* et hybrides interspécifiques: marcottage, greffage et bouturage;
Swietenia macrophylla.
Diverses espèces locales...

- **Depuis Mai 1997**: * **Spécialiste de la multiplication végétative et du clonage conforme de génotypes sélectionnés au sein du programme Arbres et Plantations du CIRAD-Forêt.**

II. ENCADREMENT SCIENTIFIQUE ET TECHNIQUE

La liste des personnes encadrées sur le plan scientifique et technique figure dans l'ordre chronologique ci-après, en précisant pour chacune le nom et le prénom, le statut social, le niveau d'études, la période, le lieu et la thématique.

DUTEL Roland, étudiant, dernière année de BTS forestier, Mars à Juin 1987, pépinière expérimentale AFOCEL de Marvejols (Lozère), régénération à partir de semis, de *Sequoiadendron giganteum* de différentes origines génétiques.

FERRET Bernard, étudiant, dernière année de BTS forestier, Mars à Juin 1987, pépinière expérimentale AFOCEL de Marvejols (Lozère), régénération à partir de semis, de *Calocedrus decurrens* de différentes origines génétiques.

GOUBIER Pascal, étudiant, dernière année de BTS forestier, Mai à Juillet 1987, pépinière expérimentale AFOCEL de Marvejols (Lozère), bouturage herbacé de *Metasequoia glyptostroboîdes*.

PEZET Catherine, étudiante, dernière année d'IUT de Biologie, Juillet à Août 1987, pépinière expérimentale AFOCEL de Marvejols (Lozère), bouturage herbacé de *Metasequoia glyptostroboîdes*.

DUMAS Elisabeth, technicienne à l'Association Forêt Cellulose (AFOCEL), première année de BTS de chimie, Avril 1988 à Février 1991, station de biotechnologies AFOCEL de l'Etançon (Seine et Marne), microgreffage et microbouturage de *Pinus pinaster*.

ROBERT Solange, étudiante, dernière année de l'ENITH, Avril à Août 1989, station de biotechnologies AFOCEL de l'Etançon (Seine et Marne), enracinement de microboutures de *Pinus pinaster*.

SCHNEIDER Joan, étudiante, Bachelor of Science, Février et Mars 1991, Septembre 1991, Mai 1992, Laboratoire du département forestier de l'université du Minnesota (U.S.A.), microgreffage et culture de méristèmes de *Pinus strobus*.

HAZARD Laurent, Coopérant Service National (CSN), D.E.A., Avril à Décembre 1991, Unité Plant Improvement and Seed Production, Luasong, Sabah, Malaisie, multiplication végétative de *Tectona grandis*, *Acacia mangium*, *Octomeles sumatrana*, *Anthocephalus chinensis* et *Endospermum peltatum*.

POUPARD Christophe, Coopérant Service National (CSN), ingénieur du GREF, Janvier 1992 à Mars 1993, Unité Plant Improvement and Seed Production, Luasong, Sabah, Malaisie, multiplication végétative de *Tectona grandis*, *Acacia mangium*, et *Kaya ivoirensis*.

McGILL Gail, étudiante, Bachelor of Science, Mai 1993 et Mai 1994, Laboratoire du département forestier de l'université du Minnesota (U.S.A.), microgreffage et culture de méristèmes de *Pinus strobus*.

VALLAURI Daniel, Coopérant Service National (CSN), D.E.A., Mars 1993 à Juillet 1994, Unité Plant Improvement and Seed Production, Luasong, Sabah, Malaisie, multiplication végétative de *Tectona grandis* et *Acacia mangium*.

BONAL Damien, Coopérant Service National (CSN), Ingénieur des Travaux Agricoles et Master of Forestry, Septembre 1994 à Décembre 1995, Unité Plant Improvement and Seed Production, Luasong, Sabah, Malaisie, multiplication végétative par bouturage, enracinement et acclimatation de vitroplants de *Tectona grandis*, micropropagation de *Acacia mangium*.

GOH Doreen, responsable pour Innoprise Corporation Sdn Bhd du Plant Biotechnology Laboratory, PhD en biologie moléculaire, Septembre 1995 à Mars 1996, Plant Biotechnology Laboratory, Tawau, Sabah, Malaisie, micropropagation de *Calamus spp.*, *Tectona grandis* et *Acacia mangium*.

DEWASME Coralie, étudiante, Licence de Physiologie Végétale Appliquée, Juin à Août 1998, Laboratoire de Génétique du CIRAD-Forêt (Baillarguet, Hérault), micropropagation de clones hybrides *Acacia mangium X Acacia auriculiformis* et *Eucalyptus urophylla X Eucalyptus grandis*; essai de caractérisation par marquage moléculaire.

CAMBILLAU Laurence, étudiante, DEUG Sciences de la Vie, Mars à Juillet 1999, Laboratoire de Génétique du CIRAD-Forêt (Baillarguet, Hérault), aptitude *in vitro* à la multiplication et à la rhizogenèse adventive de clones hybrides *Acacia mangium x Acacia auriculiformis*; utilisation des marqueurs moléculaires à des fins de caractérisation du matériel végétal.

III. EXPERTISES

- 6 - 14 Mai 1989: **Unité d'Afforestation Industrielle du Congo - UAIC -** (Congo): "Remédier aux problèmes de perte d'aptitude au clonage conforme et de variabilité intra-clonale résultante dans les plantations industrielles d'Eucalyptus"

- 17 Février - 2 Mars 1991: **Université du Minnesota, Laboratoire du Prof. W.P. HACKETT (Minnesota, USA)**: "Rajeunissement d'arbres âgés en vue de leur clonage conforme, via les techniques de culture in vitro"

- 29 Août - 6 Sept. 1991: **Université du Minnesota, Laboratoire du Prof. W.P. HACKETT** (Minnesota, USA): "Rajeunissement d'arbres âgés en vue de leur clonage conforme, via les techniques de culture in vitro"

- 8 - 12 Janvier 1992: **Eurasia and United Corporation** (Indonésie): "Proposition de projet pour la production clonale industrielle de Kapokiers"

- 14 - 18 Janvier 1992: **Agency for Forest Research & Development** (Indonésie): "Perspectives en matière d'Amélioration des espèces forestières proposées"

- 27 Avril - 1 Mai 1992: **Forest Research & Development Centre (FRDC Bogor)** et **Perum Perhutani** (Indonésie): "Perspectives en matière d'Amélioration génétique, sylviculture et techniques de propagation des espèces forestières proposées"

- 12 - 22 Mai 1992: **Université du Minnesota, Laboratoire du Prof. W.P. HACKETT** (Minnesota, USA): "Rajeunissement d'arbres âgés en vue de leur clonage conforme, via les techniques de culture in vitro"

- 16 - 27 Novembre 1992: **Philippine Council for Agriculture, Forestry and Natural Resources Research and Development (PCARRD)** (Philippines): "Mission d'identification d'un projet de Biotechnologies Forestières aux Philippines"

- 8 - 10 Mars 1993: **Soon Hua Seng Group** (Thaïlande): "Mission de prospection concernant une éventuelle collaboration en matière de propagation végétative horticole et *in vitro*, plus spécialement pour *Eucalyptus camadulensis*"

- 10 - 24 Avril 1993: **Philippine Council for Agriculture, Forestry and Natural Resources Research and Development (PCARRD), Paper Industry Corporation of the Philippines (PICOP) et Provident Tree Farm Inc (PTFI)** (Philippines): "1ère mission d'identification d'un projet de Biotechnologies Forestières aux Philippines"

- 4 - 14 Mai 1993: **Université du Minnesota, Laboratoire du Prof. W.P. HACKETT** (Minnesota, USA): "Rajeunissement d'arbres âgés en vue de leur clonage conforme, via les techniques de culture in vitro"

- 18 - 30 Octobre 1993: **Philippine Council for Agriculture, Forestry and Natural Resources Research and Development (PCARRD), Department of Environmental and Natural Resources (DENR), FAO-FORTIP Regional Meeting** (Philippines): "2ième mission d'identification d'un projet de Biotechnologies Forestières aux Philippines"

- 15 - 20 Novembre 1993: **PT. ITCI Hutani Manunggal (PT IHM)** (Indonésie): "Mission d'identification en vue d'une possible coopération avec le CIRAD-Forêt en matière d'Amélioration génétique d'espèces forestières à croissance rapide et de plantations industrielles"

- 20 - 23 Décembre 1993: **Forest Research Institute of Malaysia (FRIM)** (Malaisie Péninsulaire): "Mission d'identification en vue d'une possible coopération scientifique avec le CIRAD-Forêt en matière d'Amélioration génétique d'espèces forestières de bois précieux"

- 5 - 17 Mars 1994: **Philippine Council for Agriculture, Forestry and Natural Resources Research and Development (PCARRD), Department of Environmental and Natural Resources (DENR)** et **Bukidnon Forest incorporated (BFI)** (Philippines): "3ième mission d'identification d'un projet de Biotechnologies Forestières aux Philippines"

- 17 - 27 Mai 1994: **Université du Minnesota, Laboratoire du Prof. W.P. HACKETT** (Minnesota, USA): "Rajeunissement d'arbres âgés en vue de leur clonage conforme, via les techniques de culture in vitro"

- 8 - 16 Novembre 1994: **Philippine Council for Agriculture, Forestry and Natural Resources Research and Development (PCARRD)**

(Philippines): "Mission de suivi de l'évolution du projet de Biotechnologies Forestières aux Philippines"

- 1 - 10 Décembre 1994: **Projet LIKSIN/VINAPRAM de plantations industrielles d'espèces forestières à croissance rapide pour la pâte et le papier** (Vietnam): "Evaluation du projet et intérêt de développer des plantations clonales"

- 28 Mars - 1 Avril 1995: **P.T. KEAWOOD INDUSTRIES** (Singapore/Indonesia): "Intérêt de mise en oeuvre de techniques performantes modernes pour la production de plants améliorés dans le secteur de Pekanbaru (Sumatra)"

- 1 - 5 Mai 1995: **Projet LIKSIN/VINAPRAM de plantations industrielles d'espèces forestières à croissance rapide pour la pâte et le papier** (Vietnam): "Techniques de clonage adaptées à *Acacia mangium* et *Eucalyptus sp.*"

- 21 Août - 11 Septembre 1995: **Indian Council of Forestry Research and Education (ICFRE)** (Inde): "Expertise UNDP/FAO dans le domaine de la Culture In Vitro et Biotechnologies associées pour les espèces forestières."

- 13 - 23 Décembre 1995: **Philippine Council for Agriculture, Forestry and Natural Resources Research and Development (PCARRD)** (Philippines): "Conseils pour l'installation d'un Laboratoire de Biotechnologies Forestières à Los Banos."

- 5 - 12 Mai 1996: **Forest Department of Sri Lanka** (Sri Lanka): "Expertise UNDP/FAO (FORTIP) dans le domaine des techniques de Multiplication Végétative adaptées aux espèces forestières d'importance majeure pour le Sri Lanka."

- 19 - 25 Mai 1996: **PT INTIDAYA AGROLESTARI (INAGRO)** (Indonésie): "Techniques de multiplication végétative in vivo et in vitro pour la propagation clonale d'espèces arborescentes fruitières et forestières."

- 11 - 16 Mars 1997: **Philippine Council for Agriculture, Forestry and Natural Resources Research and Development (PCARRD)** (Philippines): "Mission de suivi de l'évolution du projet de Biotechnologies Forestières aux Philippines"

- 26 Septembre - 3 Octobre 1997: **Ambassade de France à Kuala Lumpur** (Malaisie) **et à Jakarta** (Indonésie): "Expertise gouvernementale sur les feux d'Asie du Sud-Est"

- 28 Novembre - 7 Décembre 1997: **Forest Department of Sri Lanka** (Sri Lanka) :"Mission de formation UNDP/FAO (FORTIP) sur la multiplication végétative d'espèces forestières d'importance majeure pour le Sri Lanka."

- 8 - 21 Décembre 1997: **Projet commun Innoprise Corporation Sdn Bhd (ICSB)/Cirad-Forêt "Plant Biotechnology Laboratory" - PBL -** (Sabah, Malaisie orientale): "Récolte et conditionnement de matériel végétal à partir de tecks sélectionnés en vue de leur micropropagation clonale in vitro; appui au projet PBL"

- 22 - 25 Septembre 1998: **Philippine Council for Agriculture, Forestry and Natural Resources Research and Development (PCARRD)** (Philippines): "Mission technique pour l'installation d'un laboratoire de Biotechnologies Forestières à Los Banos."

- 25 Septembre - 8 Octobre 1998: **Communauté Européenne - CEE -, DG1B** (Vietnam): "Expertise de projets forestiers tropicaux financés par la CEE/DG1B au Vietnam."

- 8 - 17 Octobre 1998: **Philippine Council for Agriculture, Forestry and Natural Resources Research and Development (PCARRD)** (Philippines): "2ième mission d'identification pour un projet de Biotechnologies Forestières aux Philippines."

- 18 - 24 Octobre 1998: **Forest Science Institute of Vietnam, Forest Seed & Planting Material Enterprise, Haivuong joint Stock Co** - nouvel acquéreur des plantations LIKSIN-VINAPRAM -, (Vietnam): "Mission CIRAD-Forêt d'identification d'actions de collaboration au Vietnam."

- 17 - 25 Mars 1999: **Tecal Nuevo S.A. et CATIÉ** (Costa Rica) : "Expertise privée sur les techniques de multiplication et plantations industrielles de Teck."

- 24 Avril - 11 Mai 1999: **Projet commun Innoprise Corporation Sdn Bhd (ICSB)/Cirad-Forêt "Plant Biotechnology Laboratory" - PBL -** (Sabah, Malaisie orientale): "Mission d'appui scientifique et technique aux projets PBL et PISP"

IV. CONGRÈS SCIENTIFIQUES INTERNATIONAUX

- 16 - 20 Septembre 1985: **Symposium: "In vitro problems related to mass propagation of horticultural plants."** Gembloux, Belgique.
  Poster, résumé et publication.

- 23 Mai 1986: **3rd Meeting of Belgian Plant Tissue Culture Group**. Izel-sur-Semois, Belgique.
  Poster.

- 10 - 14 Août 1987: **Symposium: "Plant micropropagation in horticultural industries."** Arlon, Belgique.
  Poster, publication et communication orale.

- 19 - 21 Septembre 1988: **Symposium Moet-Hennessy 1988: "Les stratégies de sélection face aux technologies modernes."** Versailles, France.
  Poster et publication.

- 25 - 30 Septembre 1988: **Symposium International sur la Physiologie des Arbres Forestiers**. Nancy, France.
  Poster, résumé et publication.

- 28 Novembre - 3 Décembre 1988: **IUFRO - Thailand Conference: "Breeding Tropical Trees."** Pattaya, Thaïlande.
  Publication et communication orale.

- 25 Juin - 8 Juillet 1989: **Nato Advanced Study Institute on Molecular Basis of Plant Aging.** Oviedo, Espagne.
  Publication et communication orale.

- 24 - 25 Octobre 1989: **Xe Colloque IAPTC: "Cinquantenaire de la culture in vitro chez les végétaux."** Versailles, France.
  Poster et publication.

- 24 - 29 Juin 1990: **VIIe International Congress on Plant Tissue and Cell Culture.** Amsterdam, Pays-Bas.
  Poster et communication orale.

- 14 -18 Septembre 1992: **IUFRO - AFOCEL Symposium: "Mass Production Technology for Genetically Improved Fast Growing Forest Tree Species."** Bordeaux, France.
  Publication et communication orale.

- 1 - 9 Décembre 1992: **FAO - UNDP Regional Symposium: "Recent Advances in Mass Clonal Multiplication of Forest Trees for Plantation Programmes."** Bogor, Indonésie.
  Publication et communication orale.

- 28 Novembre - 1 Décembre 1994: **BIO-REFOR / IUFRO-SPDC 4th International Workshop: "Bio-Re/afforestation in the Asia-Pacific Region"**. Kangar, Perlis, Malaisie.
  Publication et communication orale.

- 26 - 29 Janvier 1999: **Regional Seminar on "Site, Technology and Productivity of teak Plantations."** Chiang Mai, Thaïlande.
  Publication et communication orale..

V. LISTE EXHAUSTIVE DES PUBLICATIONS

MONTEUUIS O., 1984: Etude de la multiplication végétative de *Sequoiadendron giganteum* en vue du clonage. D.E.A. Univ. Blaise Pascal, Clermont-Ferrand, 30p.

MONTEUUIS O., 1985: Les Cyprès. AFOCEL-ARMEF - Informations-Forêt, 262, 13-24

MONTEUUIS O., 1985: La multiplication végétative du séquoia géant en vue du clonage. Annales AFOCEL 1984, 139-171

MONTEUUIS O., 1986: Microgreffage de points végétatifs de *Sequoiadendron giganteum* Buchholz séculaires sur de jeunes semis cultivés in vitro. C.R. Acad. Sc. Paris, 302, (III), 223-225

MONTEUUIS O., BON M.C., **1986**: Microbouturage du séquoia géant. Annales AFOCEL 1985, 49-87

MONTEUUIS O., 1987: Profils méristématiques de séquoias géants (*Sequoiadendron giganteum* Buchholz) jeunes et âgés durant les stades de repos végétatif et de débourrement. C.R. Acad. Sc. Paris, 305, (III), 715-720

MONTEUUIS O., 1987: In vitro meristem culture of juvenile and mature *Sequoiadendron giganteum*. Tree Physiol., 3, 265-272

MONTEUUIS O., 1987: Microgreffage du séquoia géant. Annales AFOCEL 1986, 39-61

MONTEUUIS O., 1987: *Metasequoia glyptostroboïdes*. Dans: Les Ressources Génétiques Forestières en France. Tome 1 - Les Conifères - INRA-BRG, 236p.

MONTEUUIS O., BAILLY A., **1987**: Le bouturage des cyprès. AFOCEL-ARMEF - Informations-Forêt, 313, 41-50

MONTEUUIS O., PAGES C., **1987**: Données sur le bouturage du pin sylvestre. AFOCEL-ARMEF - Informations-Forêt, 321, 143-153

MONTEUUIS O., PAGES C., SARRAN P., **1987**: De l'amélioration des conditions de bouturage en cascade du *Sequoia sempervirens*. Annales AFOCEL 1986, 111-130

MONTEUUIS O., BON M.C., BERTHON J.Y., **1987**: Micropropagation aspects of *Sequoiadendron giganteum* juvenile and mature clones. Acta Hort., 212, 489-497

MONTEUUIS O., GENDRAUD M., **1987**: Nucleotide and nucleic acid status in shoot tips from juvenile and mature clones of *Sequoiadendron giganteum* during rest and growth phases. Tree Physiol., 3, 257-263

MONTEUUIS O., BON M.C., **1987**: Enracinement et acclimatation de vitro-plants forestiers. C.R. Symposium "Plant micropropagation in horticultural industries", Florizel 87, 160-169

BON M.C., **MONTEUUIS O., 1987** Application de la technique micro 2D PAGE au microgreffage de *Sequoiadendron giganteum* Buchholz. C.R. Acad. Sc. Paris, 224, 667-670

MONTEUUIS O., 1988: Aspects du clonage de séquoias géants jeunes et âgés. Thèse de Doctorat d'Etablissement, Univ. Blaise Pascal, Clermont-Ferrand, 190p.

MONTEUUIS O., SARRAN P., DUTEL R., **1988**: Production de semis de séquoia géant en pépinière forestière moderne. AFOCEL-ARMEF - Informations-Forêt, 336, 49-63

MONTEUUIS O., FERRET B., SARRAN P., **1988**: Production de calocèdres à parti de semis. AFOCEL-ARMEF - Informations-Forêt, 342, 151-162

MONTEUUIS O., GOUBIER P., PAGES C., PEZET C., SARRAN P., **1988**: *Metasequoia glyptostroboïdes*: renseignements spécifiques et bouturage. Annales AFOCEL 1987, 211-253

MONTEUUIS O., 1989: Analyses microscopiques de points végétatifs de *Sequoiadendron giganteum* jeunes et âgés durant le repos végétatif et lors du débourrement. Bull. Soc. Bot. Fr., 136, Lettres Bot. (4/5), 317-326

MONTEUUIS O., 1989: Méristèmes, vieillissement et clonage d'arbres forestiers. Annales AFOCEL 1988, 7-39

MONTEUUIS O., 1989: Maturation concept and possible rejuvenation of arborescent species. Limits and promises of shoot apical meristems to ensure successful cloning. Dans: "Breeding Tropical Trees : Population Structure and Genetic Improvement Strategies in Clonal and Seedling Forestry". Proc. Conference IUFRO, Pattaya, Thailand, 28 Nov.-3Dec. 1988, 106-118

MONTEUUIS O., BON M.C., **1989**: Rejuvenation of a 100-year-old giant sequoia through in vitro meristem culture. Ann. Sci. For., 46, suppl.,183s-186s

MONTEUUIS O., GENESTIER S., **1989**: Analyse cytophotométrique comparée des parois du mésophylle de feuilles de *Sequoiadendron giganteum* jeunes et âgés. Bull. Soc. Bot. Fr., 136, Lettres Bot. (2), 103-107

DUMAS E., FRANCLET A., **MONTEUUIS O., 1989**: Microgreffage de méristèmes primaires caulinaires de pins maritimes (*Pinus pinaster* Ait.) âgés sur de jeunes semis cultivés in vitro. C.R. Acad. Sc. Paris, 309 (III), 723-728

MONTEUUIS O., 1990: Notes de voyage en Thaïlande. AFOCEL-ARMEF - Informations-Forêt, 396, 249-268

MONTEUUIS O., BON M.C., **1990**: Phase change in *Sequoiadendron giganteum*. Dans: Plant Aging : Basic and Applied Approaches, Plenum Press, New-York, 377-382

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VI. RÉSUMÉ DES ACTIVITÉS DE RECHERCHE

Les travaux de Recherche entrepris visent à analyser les facteurs physiologiques limitant l'efficacité de la multiplication végétative, afin de tirer le meilleur profit des techniques de propagation asexuée pour améliorer la qualité génétique des plants forestiers produits à des fins de reboisements industriels.

Le bouturage horticole permet d'évaluer les capacités rhizogènes des boutures, condition *sine qua non* de réussite, en tachant de distinguer l'influence respective des facteurs endogènes et des paramètres environnementaux. De nombreux essais sur différentes espèces arborescentes ont révélé en effet que les taux d'enracinement étaient susceptibles de varier considérablement en fonction de la période de bouturage, du niveau de prélèvement intra-individu, de l'identité génétique du matériel végétal et surtout de son âge.

Ces aspects ont été plus spécifiquement mis en évidence sur *Sequoiadendron giganteum*, dans le cadre de la thématique de recherche d'un Diplôme d'Etudes Approfondies, suivi d'une thèse de Doctorat d'Etablissement, sur le clonage de cette espèce à travers différentes techniques de propagation végétative.

Le greffage de portions végétatives plus réduites sur des porte-greffes juvéniles stimule globalement les potentialités organogènes du matériel âgé greffé, confirmant certaines présomptions ou hypothèses en la matière.

La poursuite du travail en conditions *in vitro* a permis de mieux contrôler les paramètres environnementaux, tout en travaillant sur des structures végétatives de plus en plus réduites. La mise au point d'une méthodologie de microbouturage adaptée à la micropropagation perpétuée de génotypes juvéniles et âgés s'inscrit dans cette optique. Les observations inhérentes indiquent une influence très nette de l'état physiologique, variable au cours du temps, et du degré de maturité des explants sur leurs potentialités organogènes. Les cas de rajeunissements constatés - par référence au témoin juvénile - au fil des subcultures *in vitro*, bien que plus prononcés qu'en conditions horticoles, demeurent éphémères et partiels. La possibilité de miniaturiser les explants s'est concrétisée par la mise au point d'une technique originale de microgreffage de points végétatifs caulinaires de 200 à 400μm (0,2 à 0,4mm) sur de jeunes semis *in vitro*. Parallèlement, plusieurs milliers de méristèmes primaires caulinaires ont été introduits *in vitro* sur des milieux de culture synthétiques après en avoir défini la composition et la méthodologie adaptées.

Des diverses techniques de propagation végétative appliquées, seule la culture *in vitro* de méristèmes excisés a conduit à l'obtention d'une lignée "mériclonale" - issue d'un seul méristème - de plantes rajeunies, à partir d'explants prélevés lors du débourrement sur le génotype centenaire d'origine. Le caractère probant de ce rajeunissement a pu être confirmé au niveau morphologique, organogénique et attesté au niveau biochimique par le marqueur de juvénilité "J16". De récentes informations indiquent que cette lignée rajeunie se comporte toujours, après plusieurs années en conditions *in vitro* ou après acclimatation *ex vitro* , comme le témoin juvénile.

La complexité et les multiples aspects des phénomènes de changement de phase des espèces arborescentes, considérés principalement par rapport à leur incidence sur les capacités organogéniques, nous ont incités à développer des moyens d'investigation multi-disciplinaires. Cette démarche, à partir d'un matériel expérimental bien défini, avait pour but d'identifier des indices fiables nécessaires à une bonne caractérisation du phénomène de maturation. En fonction de leur nature, ces marqueurs pourraient ensuite guider la recherche de l'origine du phénomène, de son déterminisme.

Les premières analyses biochimiques - peroxydases, polyphénols, nucléotides - ont confirmé les présomptions basées sur des observations morphologiques et organogéniques, à savoir l'existence d'une grande hétérogénéité dans l'espace et dans le temps. Le caractère éminemment fluctuant de certains composés réfute objectivement leur fiabilité en tant que marqueur du degré de maturité. La prise de conscience de cette variabilité, et l'adoption de protocoles expérimentaux définis en conséquence, ont permis de constater, lors du débourrement, l'existence de similitudes flagrantes au niveau des extrémités caulinaires végétatives entre le matériel juvénile et âgé. Ces similitudes ont pu être observées pour plusieurs critères.

L'ensemble de ces résultats, dont le plus démonstratif demeure l'obtention de la lignée mériclonale rajeunie, tend à créditer l'hypothèse selon laquelle la miniaturisation peut être considérée comme "une condition nécessaire mais non suffisante pour le rajeunissement". Ceci du fait de "fenêtres" ou "créneaux" "d'état de compétence physiologique" d'autant plus restreints d'un point de vue spatio-temporel que le sujet "tête de clone" est développé, donc âgé.

La poursuite du travail sur d'autres conifères tels que *Pinus pinaster* et *Sequoia sempervirens* a contribué à élargir la vision de la thématique à d'autres espèces. Dans le même esprit, la méthodologie du microgreffage *in vitro* a pu être adaptée avec succès à *Pseudostuga menziesii*, *Picea abies* et *Pinus pinaster*. Chez cette dernière espèce, le degré de maturité de plants régénérés par différentes vitrométhodes a pu être analysé à partir d'indices morphologiques.

L'approche analytique multi-disciplinaire du phénomène de changement de phase orientée sur les extrémités apicales, et les compétences technologiques développées à cette fin, sont à l'origine de la collaboration avec l'équipe du Professeur HACKETT de l'Université du Minnesota (U.S.A.) sur la thématique du rajeunissement de *Pinus strobus*. Ce projet a notamment favorisé la mise au point de techniques de microgreffage *in vitro* novatrices, tant sur le plan

conceptuel que technique, non publiées, contrairement aux travaux sur la culture *in vitro* de méristèmes. La meilleure réactivité en culture, là encore, est obtenue lorsque les méristèmes sont prélevés durant le débourrement.

Les travaux sur la thématique de l'aptitude à la multiplication végétative en relation avec le degré de maturité du matériel végétal sélectionné se sont poursuivis en zone subéquatoriale, sur des espèces forestières tropicales. De nombreux essais de pépinière ont confirmé pour des essences de reboisement telles que le Teck (*Tectona grandis*), le bien-fondé de réduire le développement de l'appareil caulinaire par rapport au système racinaire pour favoriser la rhizogenèse adventive, permettant la propagation clonale par bouturage industriel de génotypes âgés. Par ailleurs, l'hétéroblastie d'*Acacia mangium* a été mise à profit pour tester dans quelle mesure la morphologie foliaire pouvait être considérée comme un indice fiable de l'aptitude à l'enracinement de cette espèce. Des essais de greffage *in vivo* et de microgreffage *in vitro* menés en parallèle sur le même matériel ont montré l'intérêt, en termes de taux de réussite, de la miniaturisation du greffon lorsque celui-ci provient de sujets âgés. Les conditions de culture *in vitro* ont été surtout mises à profit pour développer une technique de micropropagation industrielle de clones de Teck âgés, introduits en conditions axéniques sous forme d'explants mononodaux ou de méristèmes. Afin de garantir le maximum de conformité clonale, la micropropagation par bourgeonnement axillaire a été privilégiée.

Cette voie n'était pas envisageable pour les espèces de rotins monocaules à intérêt commercial majeur pour le projet. Nous nous sommes dans ce cas précis intéressés aux possibilités offertes par l'embryogenèse somatique, qui a pu être obtenue à partir de sujets juvéniles. Des études histo-cytologiques ont confirmé l'origine embryogénique et somatique des plantules régénérées, dans le cas de *Calamus manan* notamment.

Dès l'origine, des collaborateurs d'origines diverses ont été associés aux travaux de recherches. Les nouvelles connaissances ont été mises à profit au fur et à mesure de leur acquisition pour orienter un certain nombre d'étudiants stagiaires ou de techniciens vers des pistes de recherches novatrices, tout en bénéficiant de leur vision spontanée de la problématique. Cette démarche a été appliquée à différentes espèces et diverses technologies, en fonction des contextes, aussi bien en France, et aux USA qu'au Sabah. Une attention toute particulière a été également portée à la valorisation, sous forme de publications en commun, des résultats novateurs obtenus.

Mots clés: **âge, bouture, changement de phase, clonage, culture *in vitro*, espèce forestière, marqueur, méristème, microgreffage, multiplication végétative, rajeunissement**.

VII. SYNTHÈSE

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1. INTRODUCTION

L'intérêt de la multiplication végétative des espèces forestières à des fins de recherche ou appliquées est universellement reconnu (Monteuuis 1988, 1989a). Cette forme de propagation asexuée permet de dupliquer, théoriquement de manière indéfinie, des génotypes sélectionnés. Elle peut s'appliquer, en fonction des situations, à un mélange d'individus "en vrac", sans distinguer les génotypes, ou à la propagation clonale de génotypes bien individualisés (Monteuuis 1985, Monteuuis et Nasi 1992, Nasi et Monteuuis 1992, Monteuuis 1993, Nasi et Monteuuis 1993).

Plusieurs techniques de multiplication végétative sont envisageables en fonction de la réactivité organogène du matériel végétal convoité, en conditions horticoles ou de culture *in vitro*, comme illustré Figure 1.

Les principaux résultats personnels obtenus dans le domaine de la multiplication végétative de différentes espèces forestières de régions tempérées et tropicales forment la base du présent document.

Dans un deuxième temps, ces résultats constituent les éléments de la discussion axée sur les phénomènes de changements phase et la compétence à la reproduction végétative chez les Arbres.

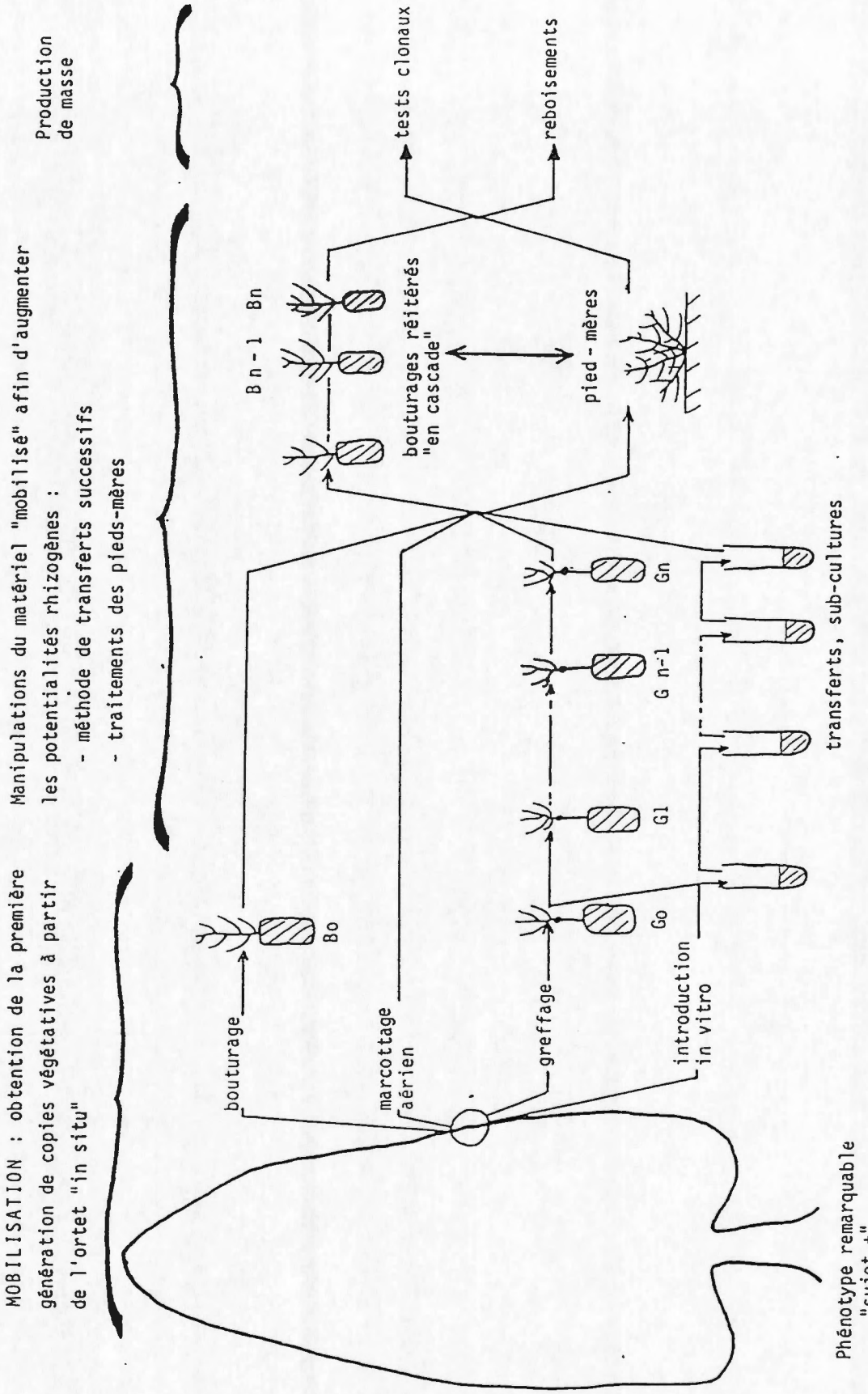

Figure 1. Schéma récapitulatif des principales techniques de multiplication végétative applicables à un individu sélectionné, encore appelé "tête de clone" ou "ortet", sans présager de son âge, et sans détailler les techniques de micropropagation in *vitro* (adapté de Monteuuis 1985).

2. PRINCIPAUX RÉSULTATS DE MULTIPLICATION VÉGÉTATIVE

21. TECHNIQUES HORTICOLES DE MACROPROPAGATION

211. Bouturage

Le bouturage est une technique de multiplication végétative horticole dont le succès est subordonné à la néoformation de racines adventives à partir d'un fragment de pousse - "ramet" pour les forestiers - prélevé sur un individu d'origine poussant *in situ* - "ortet" pour les forestiers - ou d'un pied-mère spécialement cultivé à cette fin.

Cette aptitude à la rhizogenèse adventive peut varier en fonction de facteurs propres au matériel végétal considéré, ou facteurs "endogènes", et de facteurs extérieurs, dits "exogènes", susceptibles d'interagir. Ces aspects ont été analysés sur différentes espèces.

Influence de facteurs endogènes

Génotype

L'influence du génotype, autrement dit l'"effet clonal", sur le taux d'enracinement des boutures a été mise en évidence chez plusieurs espèces de conifères de régions tempérées, plus précisément:

Sequoiadendron giganteum (Monteuuis 1985), *Cupressus arizonica* (Monteuuis et Bailly 1987), *Sequoia sempervirens* (Monteuuis et al 1987b) et *Metasequoia glyptostroboïdes* (Monteuuis et al 1988). Ce même effet s'exerce aussi sur des espèces feuillues du Sud-Est Asiatique telles que: *Anthocephalus chinensis* (Monteuuis 1993) et *Tectona grandis,* affectant également le nombre et la longueur des racines néoformées par bouture enracinée chez cette dernière espèce (Monteuuis et al 1995b)

Etat physiologique

Les différences de résultats au bouturage observées chez plusieurs espèces en fonction de la date de prélèvement des boutures suggèrent une influence non négligeable de l'état physiologique du matériel végétal sur ses capacités à la rhizogenèse adventive (Monteuuis 1985, Monteuuis et al 1987b, Poupard et al 1994, Monteuuis et al 1995a). Cette hypothèse est d'autant plus fondée que le matériel végétal se trouve dans des stades d'activité physiologique différents, par exemple repos végétatif et débourrement, ou croissance active (Monteuuis et Bailly 1987, Monteuuis et Pagès 1987, Monteuuis et al 1988).
Dans les conditions expérimentales précisées, il est vraisemblable que les paramètres environnementaux aient également un effet, en fonction notamment des variations saisonnières mentionnées ci-après.

Age

L'incidence négative de l'âge croissant de l'individu - la germination de la graine constituant l'origine temporelle de référence pour les sujets issus de semis - sur lequel sont prélevées les boutures sur leur taux d'enracinement a été confirmée chez *Sequoiadendron giganteum* (Monteuuis 1985) et *Acacia mangium* (Poupard et al 1994, Monteuuis et al 1995a). L'effet sur le nombre et la longueur des racines néoformées est moins évident, chez cette dernière espèce du moins (Poupard et al 1994, Monteuuis et al 1995a).

Niveau de prélèvement intra-individu

Les études de bouturage menées sur *Sequoiadendron giganteum* (Monteuuis 1985) et *Pinus sylvestris* (Monteuuis et Pagès 1987) notamment, ont permis de vérifier que les boutures prélevées le plus près possible de l'appareil racinaire de l'individu d'origine s'enracinent mieux que celles provenant de niveaux de prélèvement supérieurs, plus éloignés du pôle racinaire.
Cette influence du niveau de prélèvement des boutures sur le taux d'enracinement a été affinée relativement à l'ordre de ramification à partir de jeunes plants de *Metasequoia glyptostroboïdes* (Monteuuis et al 1988).

Position intra-raméale

Les boutures de "bois sec" de *Metasequoia glyptostroboïdes* (Monteuuis et al 1988) et les boutures "en vert" *d'Anthocephalus chinensis* (Monteuuis 1993) provenant de la partie apicale des rameaux s'enracinent dans de plus faibles proportions que leurs homologues de la partie proximale. Chez *Octomeles sumatrana* et *Endospermum peltatum*, les résultats chiffrés exprimant cette même tendance peuvent être controversés du fait de la mortalité importante des boutures "de tête" terminales, dans les conditions de bouturage stipulées, par rapport aux boutures "de noeud" sous-jacentes (Monteuuis 1993). Le fait que cette mortalité des boutures terminales s'atténue au fur et à mesure de l'amélioration de l'aptitude au bouturage du matériel végétal "mobilisé", et que ces boutures de tête s'enracinent finalement préférentiellement aux autres traduit bien le caractère endogène du phénomène (Monteuuis 1993).
Chez *Acacia mangium* au contraire, on observe un gradient d'enracinement intra-raméal croissant acropète en ce qui concerne les proportions d'enracinement et le nombre moyen de racines néoformées par bouture enracinée pour des semis de 6 mois des individus de 6 ans recépés (Poupard et al 1994).
L'aptitude supérieure à l'enracinement des boutures "de tête" avec bourgeon terminal par rapport aux boutures "de noeud" a été mise en évidence également chez *Sequoia sempervirens*, surtout pour les pousses orthotropes (Monteuuis et al 1987b) et chez *Tectona grandis* (Monteuuis et al 1995b).

Morphologie des pousses

Chez *Sequoiadendron giganteum*, les boutures s'enracinent d'autant mieux que leur morphologie foliaire s'apparente au type juvénile. Il a ainsi été possible d'établir une bonne corrélation (r=0,88) de type régression linéaire entre la longueur de la partie libre des feuilles en alène et le pourcentage d'enracinement des boutures (Monteuuis 1985).
Les boutures d'axes, sans bourgeon terminal, de *Sequoia sempervirens* provenant de pousses orthotropes se bouturent plus difficilement que celles provenant de pousses plagiotropes (Monteuuis et al 1987b).
Chez *Metasequoia glyptostroboïdes* (Monteuuis et al 1988), les rameaux à destinée caduque, bien distincts morphologiquement de leurs homologues pérennants, s'enracinent dans de plus faibles proportions et produisent moins de racines néoformées que ceux-ci (Monteuuis et al 1988).
Chez *Acacia mangium*, espèce hétéroblastique, des boutures ne portant que des phyllodes, morphologie foliaire de type mature, peuvent manifester les mêmes capacités rhizogènes que celles à morphologie de type juvénile caractérisée par des feuilles juvéniles composées (Monteuuis et al 1995a).
Enfin, chez *Tectona grandis*, la morphologie des pousses a une influence déterminante sur leur pourcentage d'enracinement, le nombre et la

longueur des racines néoformées par bouture enracinée (Monteuuis et al 1995b).

Influence de facteurs exogènes

Conditionnement du matériel végétal destiné à être bouturé.

* Le fait de placer des tronçons de branches de *Tectona grandis* âgés en conditions de bouturage stimule la production de pousses axillaires. Celles-ci présentent une aptitude à la rhizogenèse adventive significativement supérieure à celle des pousses prélevées directement sur l'ortet *in situ* (Monteuuis et al 1995b).

* Le recépage d'*Acacia mangium* matures en conditions *in situ* peut permettre la production de rejets de souches. Les boutures issues de ces rameaux présentent une capacité à l'enracinement adventif supérieur à celles prélevées dans la base du houppier d'individus de mêmes caractéristiques, et ce du point de vue du pourcentage d'enracinement, du nombre et de la longueur des racines néoformées (Monteuuis et al 1995a).

* Le prélèvement de boutures à partir de pieds-mères améliore l'aptitude à la rhizogenèse adventive du matériel végétal, par rapport au prélèvement directement sur l'ortet *in situ*. Cet effet bénéfique sur le pourcentage d'enracinement a pu être mis en évidence dès la première génération de "mobilisation", par bouturage chez *Sequoiadendron giganteum* (Monteuuis 1985), puis *Octomeles sumatrana* (Monteuuis 1993), et par greffage sur *Sequoiadendron giganteum* (Monteuuis 1985).
Le taux d'enracinement des boutures est amélioré par les opérations de tailles et autres pincements "en vert" réitérés sur des pieds-mères cultivés de façon intensive. Cela a pu être vérifié notamment chez *Octomeles sumatrana* (Monteuuis 1993) et sur *Tectona grandis,* relativement à la périodicité des opérations (Monteuuis et al 1995b)
La technique du bouturage "en cascade" (Monteuuis 1985, Monteuuis et Bailly 1987, Monteuuis et al 1987b, Monteuuis et al 1988), schématisée Figure 2, permet par ailleurs d'accroître les pourcentages d'enracinement et le nombre de racines adventives produites chez *Octomeles sumatrana* (Monteuuis 1993).

Préparation des boutures

Chez *Sequoia sempervirens*, la présence du bourgeon terminal favorise l'enracinent des boutures (Monteuuis et al 1987b).
En fonction des espèces et du type de bouturage, le nombre de bourgeons, lié à la longueur de la bouture, peut influer sur son

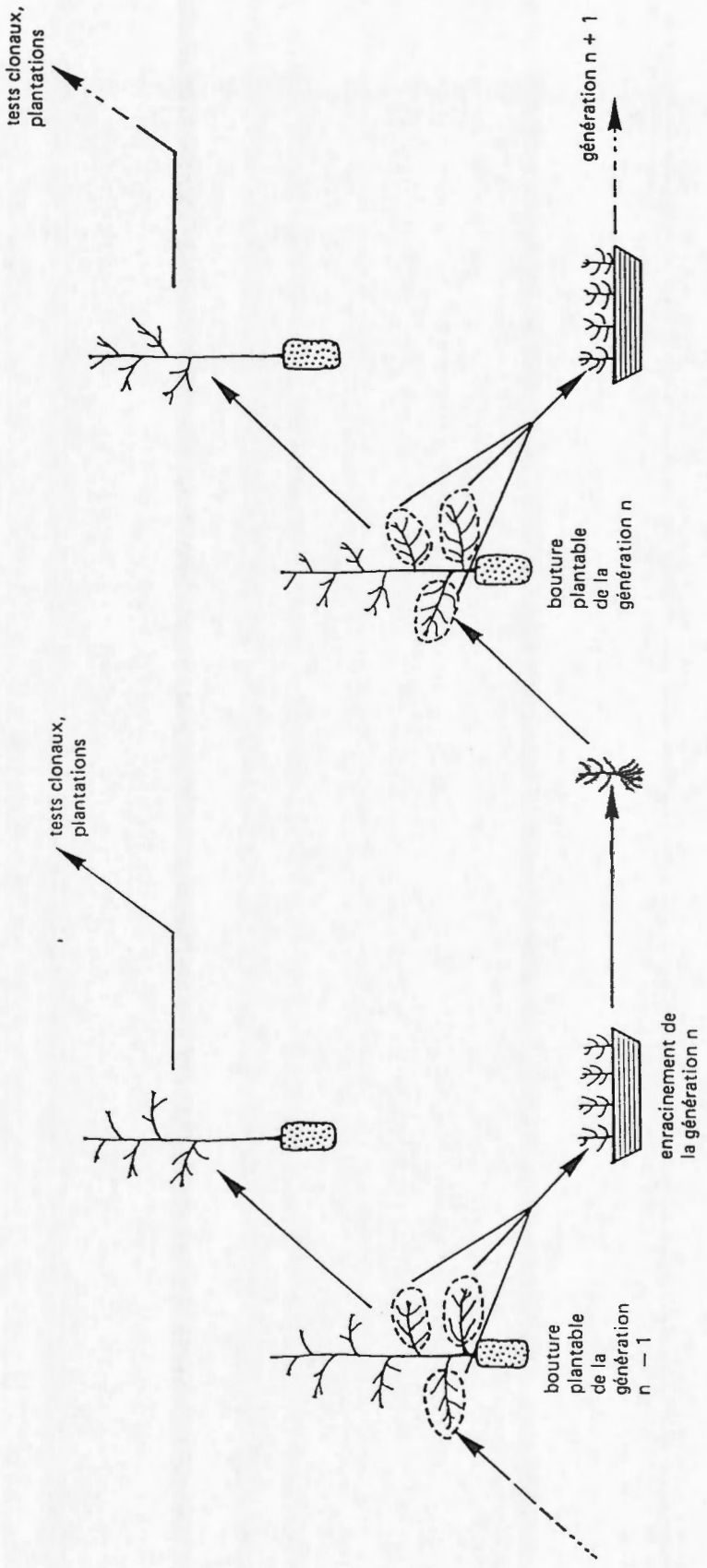

Figure 2. Schéma explicatif de la technique de bouturage "en cascade": les boutures de la génération n sont prélevées sur leurs homologues, plantables, de la génération n-1 (tiré de Monteuuis et al 1987b). Cette méthode diffère des récoltes de boutures réitérées à partir d'un seul pied-mère pratiquées classiquement.

enracinement. Ainsi, des boutures de "bois sec" de *Metasequoia glyptostroboïdes* avec 6 bourgeons, soit 3 noeuds, s'enracinent en plus grandes proportions et produisent plus de racines néoformées que leurs homologues plus courtes comptant seulement 2 ou 4 bourgeons - respectivement 1 et 2 noeuds (Monteuuis et al 1988).
Par ailleurs, le fait d'entailler superficiellement la base des boutures chez *Sequoia sempervirens* (Monteuuis et al 1987b) et *Metasequoia glyptostroboïdes* (Monteuuis et al 1988) lors de leur préparation augmente les taux d'enracinement.

Substances rhizogènes

L'effet du traitement de la base des boutures par des substances rhizogènes s'avère globalement positif sur les taux d'enracinement pour *Cupressus arizonica* (Monteuuis et Bailly 1987), *Sequoia sempervirens* (Monteuuis et al 1987b), et *Metasequoia glyptostroboïdes* (Monteuuis et al 1988). Les observations réalisées sur *Sequoia sempervirens* (Monteuuis et al 1987b) notamment, suggèrent que cet effet bénéfique peut néanmoins varier en fonction de la nature de la substance rhizogène appliquée, de l'espèce, voire du génotype (clone) et de la période de bouturage.
Sur *Endospermum peltatum*, et plus particulièrement sur *Octomeles sumatrana,* les trois composés rhizogènes appliqués ont permis d'augmenter le pourcentage d'enracinement, le nombre moyen et la longueur des racines néoformées par bouture enracinée par rapport au témoin non traité, avec des différences néanmoins selon la nature du composé (Monteuuis 1993).
L'influence bénéfique des substances rhizogènes exogènes sur le pourcentage d'enracinement et nombre moyen de racines néoformées par bouture a pu être observée chez *Acacia mangium* (Poupard et al 1994), et confirmée pour le dernier critère par une seconde étude (Monteuuis et al 1995a).

Substrat de bouturage

L'influence du substrat de bouturage sur le pourcentage d'enracinement des boutures a pu être confirmée notamment sur *Cupressus arizonica* (Monteuuis et Bailly 1987), *Pinus sylvestris* (Monteuuis et Pagès 1987) et *Metasequoia glyptostroboïdes* (Monteuuis et al 1988).

Variations saisonnières

En conditions de serre à Marvejols (Lozère), les pourcentages d'enracinement de boutures de *Pinus sylvestris* (Monteuuis et Pagès 1987), *Cupressus arizonica* (Monteuuis et Bailly 1987), *Sequoia sempervirens* (Monteuuis et al 1987b), *Metasequoia glyptostroboïdes*

(Monteuuis et al 1988) varient au cours de l'année, avec les saisons. Même en zone intertropicale (4° de latitude nord), les variations saisonnières ténues de photopériode pourraient être à l'origine des différences significatives de pourcentage d'enracinement, de nombre et de longueur de racines néoformées pour des boutures d'*Acacia mangium* (Monteuuis et al 1995a).

Interactions entre facteurs sus-cités

Génotype X Variations saisonnières

Chez *Sequoia sempervirens* (Monteuuis et al 1987b) et chez *Metasequoia glyptostroboïdes* (Monteuuis et al 1988), les différences interclonales de pourcentage d'enracinement varient en fonction de la période de bouturage, illustrant, en dépit d'analyse statistique, des interactions "génotype X variations saisonnières". Ces interactions sont particulièrement évidentes chez *Metasequoia glyptostroboïdes,* selon que l'on considère le bouturage de "bois sec" durant le repos végétatif, ou le bouturage herbacé en pleine période de végétation.

Age X Substances rhizogènes

L'interaction entre l'âge du matériel végétal et l'effet du traitement rhizogène relativement au pourcentage d'enracinement des boutures a été clairement établi chez *Acacia mangium* notamment (Poupard et al 1994).

Position intra-raméale X Conditionnement du matériel végétal

Les résultats obtenus sur *Octomeles sumatrana* indiquent une interaction entre la position intra-raméale *in situ* de la bouture et les générations successives de bouturage "en cascade" des pieds-mères, en ce qui concerne la mortalité, le pourcentage d'enracinement et le nombre de racines néoformées (Monteuuis 1993).

Morphologie des pousses X Variations saisonnières

Le pourcentage d'enracinement, le nombre et la longueur des racines néoformées observés pour trois types morphologiques bien distincts de boutures de rejets d'*Acacia mangium* varient de façon significativement différente en fonction de la période de bouturage (Monteuuis et al 1995a).

Conditionnement du matériel végétal X Substances rhizogènes

Les résultats de bouturage relatifs au pourcentage d'enracinement et au nombre de racines néoformées par bouture enracinée chez *Octomeles sumatrana* traduisent une interaction entre le conditionnement du matériel végétal d'où proviennent les boutures, et les traitements rhizogènes appliqués (Monteuuis 1993). Cette interaction a été établie de façon plus rigoureuse pour les mêmes critères d'enracinement chez *Acacia mangium* (Monteuuis et al 1995a)

Substances rhizogènes X Variations saisonnières

Les différences de pourcentage d'enracinement entre traitements rhizogènes pour des boutures de *Pinus sylvestris* (Monteuuis et Pagès 1987) et *Sequoia sempervirens* (Monteuuis et al 1987b) varient en fonction des saisons. L'interaction entre le traitement rhizogène et la période de bouturage a pu être mise en évidence en zone inter-tropicale pour des boutures d'*Acacia mangium* en ce qui concerne le taux d'enracinement et la longueur des racines néoformées (Monteuuis et al 1995a).

Substrat de bouturage X Variations saisonnières

En conditions de serre à Marvejols, les taux d'enracinement de boutures de *Pinus sylvestris* varient différemment d'un substrat de bouturage à un autre en fonction des saisons (Monteuuis et Pagès 1987).

Observations relatives au matériel bouturé

Chez certaines espèces à dimorphisme foliaire prononcé entre la phase juvénile et mature, la reprise de croissance des boutures enracinées à l'issue de la mobilisation de matériel âgé peut s'accompagner d'une réversion de la morphologie foliaire vers le type juvénile. Ce phénomène relativement fugace et variable en intensité d'une bouture à l'autre, a pu être observé notamment chez *Sequoiadendron giganteum* (Monteuuis 1984, 1985), chez *Cupressus arizonica* et *Cupressus dupreziana* (Monteuuis et Bailly 1987), et chez *Sequoia sempervirens* (Monteuuis et al 1987b). En outre, les nouvelles pousses présentent des potentialités organogènes, capacité à la rhizogenèse adventive notamment, supérieures à celles du matériel âgé d'origine. Cet aspect a été clairement mis en évidence sur *Sequoiadendron giganteum* (Monteuuis 1985) et différentes espèces tropicales (Monteuuis 1993).
L'effet bénéfique de la mobilisation par bouturage sur l'aptitude à la néoformation de racines à partir de génotypes sélectionnés âgés a

largement été mis à profit dans la pratique (Monteuuis et Bailly 1987, Monteuuis et al 1987b, Monteuuis 1993, Monteuuis et al 1995b)

212. Greffage

Le greffage peut être utilisé pour multiplier végétativement des génotypes inaptes à la rhizogenèse adventive, donc au bouturage. Il est mis à profit notamment pour la mobilisation de génotypes âgés pour lesquels les boutures ne s'enracinent pas (voir Figure 1 et Monteuuis 1985, Monteuuis et Bailly 1987, Monteuuis et Pagès 1987).

L'influence de plusieurs facteurs sur le devenir des greffons de *Sequoiadendron giganteum* et *Acacia mangium* essentiellement a été testée, donnant lieu aux observations suivantes.

Influence de différents facteurs

Génotypes greffés

Le greffage de différents génotypes de *Sequoiadendron giganteum* sur un clone de porte-greffes de *Sequoia sempervirens* se traduit par un taux de réactivité et une croissance inférieurs des greffons, par rapport aux homogreffes (Monteuuis 1985).
Le suivi de greffes de *Sequoiadendron giganteum* centenaires met en évidence des différences interclonales de cinétique d'allongement des greffons, en l'absence d'effet génotypique significatif sur les taux de reprise (Monteuuis 1984).

Age de l'ortet

L'effet négatif de l'âge croissant de l'ortet d'origine d'où proviennent les greffons sur les premières phases d'allongement des greffes a pu être observé chez *Sequoiadendron giganteum* (Monteuuis 1984).
Sur *Acacia mangium*, cet effet se ressent très nettement sur le succès au greffage, avec des résultats moyens 0% et 49% respectivement pour des individus de 3 ans et des semis de 6 mois de la même origine (Monteuuis 1995b).

Hauteur de greffage

Chez *Sequoiadendron giganteum*, la proximité de l'appareil racinaire du porte-greffe, sans effet significatif sur le taux de succès au greffage, stimule néanmoins l'allongement de greffons provenant d'individus centenaires (Monteuuis 1985).

Longueur du greffon

Toujours chez *Sequoiadendron giganteum*, nous avons pu mettre en évidence que la croissance de greffons de 4 cm de long en moyenne provenant d'individus centenaires était significativement supérieure à celle de greffons de même origine mais mesurant 7 cm en moyenne, bien que ceux-ci soient greffés plus près du système racinaire des porte-greffes que ceux-là (Monteuuis 1984, 1985).
Cet aspect a été plus particulièrement étudié à partir d'*Acacia mangium* de 3 ans pour lesquels le fait de réduire la taille du greffon de 5 à 7cm de long à 400μm fait varier les taux de réussite au greffage respectivement de 0 à 46% (Monteuuis 1995b).

Observations relatives au matériel greffé

Les observations réalisées sur *Sequoiadendron giganteum* principalement (Monteuuis 1984, 1985), et de façon plus ponctuelle sur *Cupressus dupreziana* (Monteuuis et Bailly 1987), ont permis de constater, lors de la reprise de croissance du matériel greffé, un certain rajeunissement de la morphologie foliaire par référence au témoins juvénile. L'intensité du phénomène reste très variable malgré tout d'une greffe à l'autre, et en fonction des espèces. Ainsi, chez *Acacia mangium*, les nombreuses homogreffes de matériel à morphologie mature, caractérisée par la présence exclusive de phyllodes, sur de jeunes porte-greffes n'ont pas induit une quelconque réversion de la morphologie foliaire vers le type juvénile.
Les homogreffes de mobilisation de *Sequoiadendron giganteum* centenaires sur de jeunes porte-greffes ont permis d'améliorer significativement, en sus du rajeunissement morphologique évoqué précédemment, l'aptitude à la rhizogenèse adventive des génotypes greffés (Monteuuis 1984, 1985). Ce dernier point revêt une importance particulière par rapport aux possibilités de clonage par bouturage. Par contre, le fait de réitérer l'opération "en cascade" dans les mêmes conditions n'améliore pas significativement la réactivité organogène du matériel âgé greffé, par rapport aux greffes de mobilisation de première génération (Monteuuis 1984).

22. TECHNIQUES *IN VITRO* DE MICROPROPAGATION

221. Microbouturage

Le microbouturage s'apparente dans le principe au bouturage, mais les portions d'axes végétatifs manipulées sont beaucoup plus petites - explants de 5 à 20mm généralement - et cultivées *in vitro* en conditions axéniques et environnement contrôlé. Le milieu de culture in *vitro* permet en outre la croissance des explants en l'absence de racines.

Le succès du microbouturage dépend étroitement de la mise en oeuvre de protocoles adaptés aux espèces concernées, tout en étant conscient que leur réactivité est susceptible de varier sous l'influence de facteurs endogènes.

Ces aspects sont abordés ci-après.

Mise au point de protocoles: influence de facteurs exogènes

L'étude de protocoles de microbouturage a permis d'appréhender, en sus des informations bibliographiques disponibles, les perspectives offertes dans ce domaine pour un certain nombre d'espèces arborescentes forestières. Le but est de définir des conditions de culture *in vitro* stimulant l'organogenèse, plus précisément le bourgeonnement axillaire - meilleur garant de conformité génotypique -, la croissance, et bien évidemment, en phase ultime, l'enracinement adventif dont dépend le succès à l'acclimatation (Monteuuis et Bon 1987).

Les travaux entrepris en ce sens ont permis d'établir des protocoles adaptés au microbouturage de génotypes juvéniles de âgés de *Sequoiadendron giganteum* jusqu'à la phase d'enracinement *in vitro*, plus particulièrement étudiée (Monteuuis et Bon 1986). Cet aspect a été également privilégié en ce qui concerne le microbouturage de *Sequoia sempervirens* (Bon et al 1994) et de *Pinus pinaster* (Dumas et Monteuuis 1995), où les effets bénéfiques respectifs de l'obscurité et du charbon actif, entre autres, sur la rhizogenèse *in vitro* ont été mis à profit.
Dans un autre contexte, il a paru fondé d'essayer d'améliorer les protocoles de micropropagation adaptés à *Acacia mangium* et *Paraserianthes falcataria*, vu l'importance économique de ces essences pour le Sud-Est asiatique (Monteuuis et Nasi 1992, Nasi et Monteuuis 1993). A cette fin, l'étude a privilégié l'influence des macroéléments et des régulateurs de croissance sur les capacités organogènes *in vitro*, au niveau de l'appareil caulinaire essentiellement, de ces deux espèces

(Bon et al 1998). La méthodologie en vigueur jusqu'alors avait malgré tout permis de micropropager durant trois ans des clones juvéniles et âgés d'*Acacia mangium* (Bon et Monteuuis 1996), et d'étudier l'enracinement *in vitro* de ce matériel, stimulé par l'obscurité et l'addition d'auxine dans le milieu (Monteuuis, en préparation). A ce jour, l'ensemble de ces activités est demeuré à une échelle expérimentale.
En revanche, sur *Tectona grandis*, la mise au point de protocoles in *vitro* délibérément simples en mettant à profit des installations de pépinière adaptées pour l'enracinement (Bonal et Monteuuis 1997), a engendré la production industrielle de plusieurs dizaines de milliers de vitroplants. de teck (*Tectona grandis*) au Sabah (Malaisie orientale), dans le nord de Bornéo (Monteuuis 1995a, Goh et Monteuuis 1997, Monteuuis et al 1998).

Influence de facteurs endogènes

Etat physiologique

Les variations d'état physiologique semblent constituer l'hypothèse la plus plausible pour interpréter les écarts d'accroissement en hauteur et de pourcentages d'enracinement de microboutures de *Sequoiadendron giganteum* cultivées *in vitro* en conditions d'environnement stable (Monteuuis 1985, Monteuuis et Bon 1986, Monteuuis et al 1987a). Il semble même que ces fluctuations d'allongement puissent varier en fonction de la composition minérale du milieu de culture *in vitro* (Monteuuis 1988).
Les dosages connexes de composés phénoliques réalisés accréditent l'influence de l'état physiologique du matériel micropropagé sur l'expression de ses potentialités organogènes (Bon et Monteuuis 1986, Monteuuis et al 1987a).
Les différences de capacités organogènes observées dans des conditions d'environnement stabilisées de culture *in vitro* chez d'autres espèces (Bon et al 1994, Dumas et Monteuuis 1995) sont également révélatrices, et incitent à ne pas sous-estimer l'influence vraisemblable des variations d'état physiologique sur l'aptitude à l'organogenèse du matériel végétal micropropagé.

Age

L'influence négative de l'âge croissant du matériel végétal d'origine sur la croissance *in vitro* des microboutures a pu être mise en évidence notamment chez *Sequoiadendron giganteum* (Monteuuis et Bon 1986, Monteuuis et al 1987a), en fonction malgré tout de la composition minérale du milieu de culture (Monteuuis 1988).
Cet effet inhibiteur se ressent particulièrement quant à l'aptitude à la rhizogenèse adventive chez *Sequoiadendron giganteum* (Monteuuis et Bon

1986), en liaison avec des différences sensibles de profils peroxydasiques (Monteuuis et al 1987a), et chez *Pinus pinaster* (Dumas et Monteuuis 1995). Il se traduit également par une diminution de l'aptitude à l'enracinement *in vivo* de microboutures de *Tectona grandis* issues de matériel âgé, par comparaison au matériel juvénile (Bonal et Monteuuis 1997).

Origine topographique

Les microboutures provenant de la base d'un *Sequoia sempervirens* de 90 ans environ manifestent une aptitude à l'organogenèse *in vitro* bien supérieure à leurs homologues issues du houppier (Bon et al 1994). Cette supériorité s'exprime au niveau de la croissance, du taux d'enracinement, du nombre, de la répartition spatiale et de la longueur des racines néoformées. Les deux origines se différencient également sur le plan de la morphologie, et des profils protéiques, indépendamment du fait qu'elles proviennent du même arbre, et ont donc le même génotype.

Interactions entre facteurs exogènes et facteurs endogènes

Composition minérale du milieu de culture X Origine du matériel végétal

Chez *Sequoiadendron giganteum*, les cinétiques de croissance comparées de microboutures d'un clone juvénile et d'un clone centenaire au cours de deux ans de subcultures mettent en évidence une très nette interaction entre l'origine du matériel végétal et la composition minérale du milieu de culture (Monteuuis 1988), affectant également le type morphologique (Monteuuis et Bon 1986, Monteuuis 1988).

Charbon actif X Age du matériel végétal

L'examen de plusieurs critères d'enracinement *in vitro* de microboutures de *Pinus pinaster* révèle une forte interaction entre le charbon actif contenu dans le milieu d'expression rhizogène et l'âge du matériel végétal d'origine (Dumas et Monteuuis 1995).

Observations relatives au matériel microbouturé

La plupart des espèces arborescentes microbouturées montrent une grande variabilité de réponses organogènes d'un explant à l'autre et au

cours des sub-cultures, en dépit de l'environnement stabilisé. Cette hétérogénéité a été observée entre génotypes juvéniles chez *Acacia mangium* et *Paraserianthes falcataria* (Bon et al 1998), mais également au sein de clones issus de matériel juvénile, et de façon plus spectaculaire lorsque que la tête de clone est âgée. Ceci a pu être vérifié notamment dans le cas de *Sequoiadendron giganteum* (Monteuuis et Bon 1986), *Sequoia sempervirens* (Bon et al 1994) et *Acacia mangium* (Bon et Monteuuis 1996).

Le microbouturage de matériel âgé peut, en fonction des espèces et des conditions de cultures, favoriser des rajeunissements morphologiques et physiologiques, discernables pour ces derniers sur le plan des capacités organogènes *in vitro*. Ainsi chez *Sequoiadendron giganteum*, au cours des régimes de subcultures, certaines microboutures de clones âgés peuvent évoluer vers le type morphologique juvénile, voire, dans de rares cas, s'enraciner spontanément (Monteuuis et Bon 1986). Chez *Pinus pinaster*, le fait de sub-cultiver des microboutures provenant de génotypes âgés sur des milieux particuliers favorise la production d'euphylles aptes à néoformer des bourgeons adventifs (Dumas et Monteuuis 1991).
Pour ces deux espèces, il parait nécessaire de souligner le caractère instable de ces rajeunissements, décelé en conditions *in vitro* pour *Sequoiadendron giganteum* (Monteuuis 1988), et après acclimatation en conditions *in vivo* pour *Pinus pinaster* (Monteuuis et Dumas 1992).
Relativement à ces aspects, *Tectona grandis* constitue un cas bien particulier. Même lorsqu'elles proviennent de génotypes âgés, les cultures manifestent une réactivité organogène tout-à-fait satisfaisante et relativement homogène. La phase de stabilisation des cultures ne dure que quelques mois, à laquelle succède la phase de production de vitroplants de qualité (Monteuuis 1995, Monteuuis et al 1998). Des tests de comportement au champ devrait permettre d'analyser rapidement, sur base de données chiffrées, la supériorité des plants issus de microbouturage, par rapport à ceux issus de bouturage horticole, par rapport à un échantillon témoin de semis.
Une évaluation similaire, mais limitée à une comparaison entre un clone issu du microbouturage *in vitro* d'un *Acacia mangium* de 5 ans sélectionné pour sa supériorité de croissance,et ses descendants de semis est en cours, dans le même contexte géographique. A l'issue de quelques mois de plantation "au champ", le matériel provenant d'*in vitro* révèle une homogénéité intraclonale et une croissance tout-à-fait satisfaisantes.

222. Microgreffage

La technique de microgreffage de points végétatifs d'individus âgés sur de jeunes semis de la même espèce *in vitro* a été initialement mise au point sur *Sequoiadendron giganteum* (Monteuuis 1986 et 1987a). Elle a été ensuite adaptée à d'autres espèces de conifères, précisément: *Pinus pinaster* (Dumas et al 1989), *Pinus strobus* (Goldfarb et al 1992), *Picea abies* (Monteuuis 1994), *Pseudotsuga menziesii* (Monteuuis 1995c), et à *Acacia mangium*, espèce feuillue de zone tropicale humide (Monteuuis 1995b, 1996).

Les fondements et la présentation de cette technique ont déjà été développés (Monteuuis 1987a, Monteuuis et Dumas 1990). Succinctement, le microgreffage *in vitro* de points végétatifs cumule les avantages du greffage, en réduisant les problèmes d'incompatibilité, dûs à l'âge notamment (Monteuuis 1995b), et de la culture de méristèmes.

Les essais de microgreffage *in vitro* sur endosperme et embryons zygotiques matures excisés réalisés initialement sur *Pinus pinaster* et *Pseudotsuga menziesii* (Monteuuis, résultats non publiés), puis sur *Pinus strobus* (Goldfarb et al 1992, Hackett et al 1994), et demeurés à un stade préliminaire du fait de raisons contextuelles, mériteraient d'être poursuivis.

Influence de facteurs exogènes

Taille du greffon

Sur *Sequoiadendron giganteum*, les taux de réussite au microgreffage sont nettement inférieurs pour des greffons de 0,2 à 0,3 mm par rapport à des greffons de 0,4 à 0,5mm (Monteuuis 1987a). Il en est de même pour des greffons de *Acacia mangium* de 0,15 à 0,20 mm par comparaison aux greffons de 0,4mm habituellement utilisés (Monteuuis 1996).

Technique de microgreffage

Sur *Picea abies* (Monteuuis 1994), les taux de réussite du microgreffage en "fente latérale" sur épicotyle, technique initialement mise au point sur *Sequoiadendron giganteum* (Monteuuis 1986 et 1987a), sont bien supérieurs à ceux obtenus par microgreffage en "tête". Il en est de même chez *Pseudotsuga menziesii* par rapport au microgreffage en "fente terminale", plus efficace malgré tout que le microgreffage en "tête" (Monteuuis 1995c). Chez *Acacia mangium*, au contraire, la technique de microgreffage en "fente terminale" s'avère plus efficace que le microgreffage en "fente latérale" (Monteuuis 1996).

Niveau de greffage

Le microgreffage de points végétatifs de *Sequoiadendron giganteum* sur l'épicotyle des jeunes semis porte-greffes donne de meilleurs taux de réussite que lorsque l'opération est effectuée sur l'hypocotyle (Monteuuis 1987a).

Obscurité

Le fait de placer des microgreffes de *Picea abies* 2 à 3 semaines à l'obscurité juste après le microgreffage améliore nettement les taux de réussite (Monteuuis 1994). Il en va différemment pour *Acacia mangium* (Monteuuis 1996).

Influence de facteurs endogènes

Identité spécifique

Contrairement aux autres espèces sus-citées, les tentatives d'adapter la technique de microgreffage originelle (Monteuuis 1986 et 1987a) à *Eucalyptus gunnii* et *Tectona grandis* (Monteuuis, résultats non publiés) se sont globalement soldées par des échecs associés à des phénomènes d'oxydations rapides des greffons, malgré divers traitements à l'obscurité et l'emploi d'antioxydants, .

Age de l'ortet d'origine

Chez *Pinus pinaster* (Dumas et al 1989), les observations réalisées ne nous permettent pas de mettre en évidence de différence significative des taux de réussite au microgreffage en fonction de l'âge de l'ortet d'origine. Cette constatation s'applique également à *Acacia mangium* (Monteuuis 1995b), par contraste avec les résultats de greffage *in vivo*. Mais toujours chez cette espèce, la reprise de croissance *in vitro* des greffons vivants provenant de génotypes âgés est plus aléatoire que pour ceux issus de jeunes individus.

Stade végétatif

Chez *Sequoiadendron giganteum*, les pourcentages de réussite au microgreffage pour des greffons prélevés sur du matériel en repos végétatif sont plus élevés que lorsque le matériel est en croissance (Monteuuis 1987a).

Observations relatives au matériel microgreffé

La reprise de croissance en conditions de culture in vitro des microgreffes réussies s'effectue de façon très variable d'une microgreffe à l'autre. Cette hétérogénéité de développement a été observée entre greffons prélevés *in vivo* sur le même lieu et microgreffés à la même date et dans les mêmes conditions notamment pour: *Sequoiadendron giganteum* (Monteuuis 1987a), *Pinus pinaster* (Dumas et al 1989), *Picea abies* (Monteuuis 1994), *Pseudotsuga menziesii* (Monteuuis 1995c) et *Acacia mangium* (Monteuuis 1995b et 1996). La variabilité des réponses au microgreffage s'exprime aussi bien à l'échelon inter- qu'intra-clonal pour l'ensemble des espèces pré-citées.
Lors des premières phases de développement du greffon, certains cas de rajeunissements morphologiques, par analogie au type juvénile, ont pu être observés sur *Acacia mangium* (Monteuuis 1996), espèce hétéroblastique, et au niveau intra-clonal chez *Sequoiadendron giganteum* (Monteuuis 1987a) et *Pinus pinaster* (Dumas et al 1989, Monteuuis et Dumas 1992), espèces à dimorphisme foliaire suffisamment prononcé entre la phase juvénile et le phase mature. Chez des microgreffes de séquoias géants centenaires, le rajeunissement morphologique observé correspond à une densité protéique plus importante, notamment du point de vue des protéines acides (Bon et Monteuuis 1987). Chez *Pinus pinaster*, le rajeunissement induit par le microgreffage de sujets centenaires peut s'exprimer par l'apparition d'euphylles, formations foliaires caractéristiques du stade juvénile chez les pins, aptes à néoformer des bourgeons adventifs (Dumas et Monteuuis 1991). Il convient malgré tout de souligner le caractère sporadique d'apparition de ce phénomène, variable en intensité et fréquence d'une microgreffe à une autre au sein d'un même échantillon expérimental. Ces rajeunissements s'estompent par ailleurs plus ou moins rapidement au cours de la croissance *in vitro* de la microgreffe (Monteuuis 1987a). Des greffons rajeunis de séquoia géants, sectionnés pour être introduits sur un milieu de culture à effet "rajeunissant" (Monteuuis 1988 et 1989b), retrouvent rapidement leur aspect morphologique mature originel. Ce peut être sous l'effet du charbon actif du milieu de culture, supposé adsorber toutes sortes de substances, entre autres d'hypothétiques composés "rajeunissants" endogènes transmis par le porte-greffe (Monteuuis 1988). Ces rajeunissements morphologiques peuvent encore s'exprimer en conditions *in vivo*, à l'issue de la phase d'acclimatation, du moins chez *Sequoiadendron giganteum* (Monteuuis 1987a), et, chez *Pinus pinaster,* de façon plus durable que pour des microboutures du même clone (Monteuuis et Dumas 1992). Cette observation tend à confirmer l'influence "rajeunissante" bénéfique du porte-greffe juvénile (Monteuuis 1988).

223. Culture de méristèmes

La culture de méristèmes primaires caulinaires sur des milieux de culture synthétiques en conditions *in vitro* constitue l'étape ultime du cheminement visant à reproduire végétativement des individus à partir de structures méristématiques pré-existantes (Monteuuis 1989b). Nonobstant son intérêt pour la diffusion et la conservation de matériel végétal exempt de germes phyto-pathogènes, la culture de méristèmes se justifie dans le cadre de notre problématique à travers les possibilités de réactiver le potentiel organogénique d'individus âgés en vue de leur clonage conforme.

A cette fin, la culture de méristèmes a été appliquée plus spécifiquement à *Sequoiadendron giganteum* (Monteuuis 1987c, 1988 et 1991), *Pinus pinaster* et *Pseudostuga menziesii* (Monteuuis, résultats non publiés), *Pinus strobus* (Hackett et al 1994, Goldfard et al 1996) et *Tectona grandis* (Monteuuis et al 1998, et en préparation). Pour chacune des espèces pré-citées, les méristèmes prélevés comprenaient le dôme méristématique, et le cas échéant, en fonction du plastochrone, quelques primordia foliaires. Les ébauches foliaires et jeunes feuilles étaient systématiquement excisées. La taille hors tout de ces méristèmes peut varier en fonction des espèces, de l'âge du matériel végétal et du stage végétatif, comme observé pour *Sequoiadendron giganteum* (Monteuuis 1987b et 1989c) et *Pinus strobus* (Goldfarb et al 1996). Elle avoisine généralement 0,2 à 0,3mm, qui constitue la limite inférieure imposée par les contraintes techniques de manipulations.

Mise au point de protocoles: influence de facteurs exogènes

L'influence de divers facteurs exogènes sur la culture de méristèmes des espèces pré-citées a été étudiée, donnant lieu au bilan suivant:

Concentration de la solution macrominérale

Les essais réalisés de mise en culture de méristèmes de *Sequoiadendron giganteum* (Monteuuis 1987c), *Pinus pinaster*, *Pseudotsuga menziesii* (Monteuuis, résultats non publiés), *Pinus strobus* (Goldfarb et al 1996), *Tectona grandis*, et d'apex de 200 à 300μm d'*Acacia mangium* (Monteuuis, résultats non publiés), incitent à utiliser des solutions macrominérales diluées par rapport à l'usage courant, afin que leur salinité globale soit comprise entre 20 et 30mM. Des salinités plus élevées favorisent l'apparition de malformations, de phénomènes d'hypertrophie, d'hyperhydrie et de callogenèse entraînant rapidement la dégénérescence des cultures.

Nature des macroéléments

La procédure consiste à étudier l'effet de solutions macrominérales de concentration ionique totale similaire, soit comprise entre 20 et 30mM, mais différant du point de vue de la nature des macroéléments (Monteuuis 1987c, Bon et al 1998). La nature des macroéléments a une influence significative sur le développement *in vitro* de méristèmes de *Sequoiadendron giganteum* (Monteuuis 1987c). L'effet sur des cultures de méristèmes de tecks et d'apex de *Acacia mangium* est moins marqué (Monteuuis, résultats non publiés).

Nature et concentration des régulateurs de croissance exogènes

Plus encore que les macroéléments, les régulateurs de croissance incorporés dans les milieux de culture sont susceptibles d'agir en fonction de leur nature et leur concentration. L'influence de plusieurs auxines, cytokinines et de l'acide gibbérellique à différentes concentrations a été testée plus particulièrement sur *Sequoiadendron giganteum* où l'acide naphtalène acétique (ANA) peut s'avérer bénéfique en fonction de l'âge et du stade végétatif du matériel végétal (Monteuuis 1987c). Son effet est beaucoup plus mitigé sur *Pinus strobus* lorsque les méristèmes sont prélevés au moment de la reprise de croissance (Goldfarb et al 1996). Les cytokinines paraissent plus utiles que les autres types de régulateurs de croissance pour la culture de méristèmes de *Tectona grandis* (Monteuuis, en préparation), voire pour la phase d'initiation de culture d'apex d'*Acacia mangium*, éventuellement en association avec de l'acide gibbérellique (Monteuuis, résultats non publiés), contrairement aux observations réalisées sur *Sequoiadendron giganteum* (Monteuuis 1987c).

Support de culture

La consistance du milieu de culture peut influer sur le devenir des méristèmes excisés. Le fait d'introduire ces derniers sur des amas de fibres de nitrate de cellulose imbibés de milieu liquide entraîne rapidement leur dégénérescence dans le cas de *Sequoiadendron giganteum* et *Pseudotsuga menziesii* (Monteuuis, résultats non publiés). Les milieux gélifiés ont été les plus fréquemment utilisés sur l'ensemble des espèces travaillées, bien que la nature de l'agent gélifiant puisse avoir une influence, comme cela a pu être clairement montré chez *Tectona grandis* (Monteuuis, en préparation). L'emploi d'agarose peut être un moyen d'éviter l'influence néfaste éventuelle des impuretés contenues dans les diverses géloses disponibles (Goldfarb et al 1996). Sur *Pinus strobus*, l'utilisation de filtres d'ester de cellulose entre le milieu de culture gélifié et les méristèmes a un effet tout-à-fait bénéfique sur la survie de ces derniers (Goldfarb et al 1996).

Influence de facteurs endogènes

Identité génétique

Les résultats de culture *in vitro* de méristèmes varient considérablement d'une espèce à l'autre, nécessitant la mise au point de protocoles spécifiques. La méthodologie développée dans le cas de *Sequoiadendron giganteum* (Monteuuis 1987c) a dû être modifiée pour *Pinus strobus* (Goldfarb et al 1996) et pour *Tectona grandis* (Monteuuis, en préparation), qui réagit tout-à-fait différemment aux cytokinines exogènes. Chez cette dernière espèce en outre, l'évolution des méristèmes placés en culture peut être influencée par des effets clonaux. Et les milieux de culture définis pour le teck se sont avérés non adaptés à *Acacia mangium*.

Age

L'âge du génotype d'où proviennent les méristèmes influe sur leur réactivité *in vitro*, dès la phase d'initiation. Cet aspect a été plus particulièrement étudié chez *Sequoiadendron giganteum* (Monteuuis 1987c). Les méristèmes de jeunes sujets de cette espèce se développent plus facilement, sur une gamme de milieux plus large, du point de vue de la composition minérale notamment, que leurs homologues provenant de sujets âgés. Ces derniers, globalement moins réactifs, se révèlent plus stricts quant à la composition du milieu de culture (Monteuuis 1987c, Monteuuis et al 1987a, Monteuuis et Bon 1990). Une plus grande inertie à l'organogenèse *in vitro* des méristèmes provenant de matériel âgé par comparaison avec ceux issus de jeunes génotypes a été constatée également chez *Pinus strobus* (Goldfarb et al 1996).

Stade végétatif

L'état physiologique des espèces arborescentes est éminemment variable, même au cours de la journée (Monteuuis et Bon 1986, Monteuuis et al 1987a, Monteuuis 1988). Afin d'essayer de limiter les interférences de ces fluctuations inopinées sur la réactivité organogène de cultures *in vitro* de méristèmes de *Sequoiadendron giganteum*, l'influence de la composante physiologique a été analysée par rapport à deux stades phénologiques bien distincts, à savoir le repos végétatif et la reprise de croissance caulinaire (Monteuuis 1987c). Les espèces de régions tempérées se prêtent plus à ce type d'analyse que celles de la zone intertropicale, et surtout équatoriale où les différences saisonnières sont nettement moins marquées. Les résultats afférents (Monteuuis 1987c) montrent que la reprise de croissance du matériel végétal sur lequel sont récoltés les méristèmes stimule le potentiel organogène *in vitro* de ces derniers. En outre, durant ce stade, la dépendance vis-à-vis de l'ANA contenu dans le milieu d'initiation est moins marquée.

Cet effet bénéfique de la reprise de croissance en longueur des rameaux sur la survie et sur l'activité organogène des méristèmes cultivés *in vitro*, a été confirmé très clairement aussi à partir d'un clone âgé de *Pinus strobus*, indépendamment de la concentration en ANA dans le milieu de culture (Goldfarb et al 1996).

Interactions entre facteurs exogènes et endogènes

Nature des macroéléments X Age

Les méristèmes de *Sequoiadendron giganteum* âgés mis en culture *in vitro* révèlent une affinité plus stricte vis à vis de la nature des macrominéraux que ceux provenant de matériel juvénile (Monteuuis 1987c).

Régulateurs de croissance X Age

L'effet positif de l'ANA ajouté dans le milieu de culture sur la réactivité *in vitro* des méristèmes de séquoia géant est plus marqué pour des génotypes âgés que jeunes (Monteuuis 1987c).

Régulateurs de croissance X Stade végétatif

L'influence bénéfique de l'ANA sur la réponse organogène *in vitro* de méristèmes de *Sequoiadendron giganteum* est plus probante pour du matériel végétal en repos végétatif hivernal que lors de la reprise de croissance (Monteuuis 1987c). Les observations relatives à la culture *in vitro* de méristèmes de *Pinus strobus* reflètent également la même tendance (Goldfarb et al 1996).

Observations relatives au matériel issu de culture de méristèmes

Le fait le plus marquant émergeant de ces travaux reste l'obtention chez *Sequoiadendron giganteum* d'une lignée méristématique, ou "mériclonale", rajeunie à partir d'un méristème issu d'un sujet centenaire (Monteuuis 1988). En se référant au matériel juvénile, le rajeunissement observé d'un point de vue morphologique a pu être confirmé au niveau des capacités organogènes, aptitude à la rhizogenèse adventive notamment (Monteuuis 1991), et moléculaire à travers la mise en évidence d'un polypeptide de 16 kDa, "J16"; marqueur de l'état juvénile chez cette espèce (Bon et Monteuuis 1991). Comparativement, les autres méristèmes provenant du même génotype centenaire se développent difficilement *in vitro*, en

conservant les caractéristiques du matériel âgé, tant du point de vue morphologique que des capacités organogènes, bien inférieures au matériel juvénile. Cette lignée "mériclonale" rajeunie se comporte de façon identique au matériel juvénile, issu lui aussi des mêmes conditions de culture de méristèmes, ceci au cours des phases d'acclimatation, de pépinière et ultérieurement au champ. La persistance de cet état rajeuni à l'issue de l'acclimatation est à souligner, par contraste avec le caractère instable des cas de rajeunissement observés au cours du microbouturage ou à l'issue du microgreffage (Monteuuis 1989). Cette lignée rajeunie est toujours propagée *in vitro* par microbouturage, en conservant depuis dix années les mêmes caractéristiques juvéniles.

Quelques plants issus de culture de méristèmes de *Pinus strobus* juvéniles ont été régénérés, enracinés *in vitro* et acclimatés avec succès en serre dans le Minnesota (U.S.A.) où ils se sont développés de façon satisfaisante, du moins lors des premiers stades de croissance.

De nombreuses lignées "mériclonales" ont été produites à partir de génotypes âgés de teck au Sabah, dans le nord de Bornéo, et plantées (Monteuuis et al 1998). Les conditions de croissance impressionnantes de ce matériel dans un tel environnement devraient permettre d'analyser l'hétérogénéité phénotypique éventuelle au sein de ces lignées issues d'un seul méristème, et entre elles, en fonction des différents génotypes d'origine dont elles sont issues, pour une meilleure appréhension de la notion de variabilité intra-clonale.

224. Bourgeonnement adventif

Le bourgeonnement adventif provient de la néoformation, ou formation *de novo*, de méristèmes gemmaires par des cellules de couches histologiques superficielles à l'issue d'un processus de dédifférenciation pouvant favoriser la formation d'un cal transitoire. Le bourgeonnement adventif, au même titre que l'embryogenèse somatique (Goh et al 1997, 1998), se différencie donc radicalement de par son origine histologique de toutes les autres techniques de propagation végétative considérées précédemment. Celles-ci étaient basées sur le bourgeonnement axillaire à partir de structures méristématiques préexistantes.

Les risques de variations somaclonales susceptibles de déprécier considérablement la valeur des génotypes sélectionné originellement, et ce caractère "artificiel", nécessitant un investissement matériel et temporel pour des résultats aléatoires, ont limité son application à *Pinus pinaster*. Des tigelles ont ainsi été régénérées par néoformation de bourgeons, notamment à partir d'euphylles produites par des microgreffes de génotypes centenaires, pour être ensuite enracinées in *vitro* puis acclimatées *ex vitro* (Dumas et Monteuuis 1991).

Il est intéressant de noter, toujours sur *Pinus pinaster*, qu'à l'issue d'une saison de croissance en serre, les plants produits par bourgeonnement adventif sur cotylédons présentent plus de caractères morphologiques matures que des semis *in vitro* de même âge (Monteuuis et Dumas 1992).

3. APTITUDE À LA MULTIPLICATION VÉGÉTATIVE: ESSAI DE CARACTÉRISATION

L'ensemble des observations précédentes indique une influence déterminante de l'état physiologique du fragment de végétal prélevé sur son aptitude à la multiplication végétative, surtout lorsque la rhizogenèse adventive est impliquée. Il parait dès lors fondé de rechercher des indicateurs des potentialités à la reproduction végétative, entendue clonage conforme (Monteuuis 1988). Cette recherche a été entreprise au niveau macroscopique, microscopique et biochimique.

31. APPROCHE MACROSCOPIQUE

311. Indices de maturité

La présence de fleurs ou de fruits définit, hormis dans des cas très particuliers de néoténie, l'état mature, associé généralement à un déclin du potentiel à la reproduction végétative et à la rhizogenèse adventive (Monteuuis 1988, 1989a et b). Ceci a pu être vérifié notamment chez *Sequoiadendron giganteum*, où l'ampleur du développement de l'appareil caulinaire des sujets matures permet néanmoins de mettre en évidence une zonation intra-individu quant à l'aptitude à la rhizogenèse adventive (Monteuuis 1985, 1988).

312. Morphologie foliaire

La morphologie des feuilles chez les espèces hétéroblastiques comme *Acacia mangium*, ou à dimorphisme foliaire contrasté entre le stade juvénile et le stade mature, peut être considérée comme indice de degré de maturité, et donc de potentiel à la rhizogenèse adventive, comme mis en évidence chez *Sequoiadendron giganteum* notamment (Monteuuis 1985, 1988).

L'obtention de bourgeons adventifs à partir d'euphylles apparues à l'issue d'opérations de microgreffage ou de microbouturage *in vitro* de pins maritimes âgés est également révélatrice du degré de juvénilité associé à la morphologie foliaire et à des modifications de la synthèse protéique (Bon et Monteuuis 1987, Bon et al 1994).

La variabilité des réponses organogènes constatée au cours du temps, avec des pourcentages d'enracinement *in vitro* susceptibles de varier de 0 à 100% pour des microboutures juvéniles de *Sequoiadendron giganteum* (Monteuuis et Bon 1986) incite à considérer le type foliaire juvénile comme une "condition nécessaire mais non suffisante" de la compétence à la reproduction végétative.

32. APPROCHE MICROSCOPIQUE

321. Composés pariétaux du mésophylle

La référence aux feuilles en tant qu'éventuels indicateurs du potentiel à la multiplication végétative s'est poursuivie au niveau microscopique. Des feuilles provenant de séquoias géants jeunes, caractérisés par une bonne aptitude au bouturage, et de séquoias géants âgés, inaptes à la rhizogenèse en adventive, ont été comparées du point de vue de leur teneur en composés pariétaux, et ce, en vue de rechercher des marqueurs du degré de juvénilité. Les analyses histochimiques par cytophotométrie montrent que les parois du mésophylle du matériel âgé contiennent plus de polysaccharides que celles du jeune matériel. Cette tendance, qui persiste après l'hydrolyse des substances pectiques, puis des hémicelluloses, et enfin des autres polysaccharides non cellulosiques pourrait être due à des teneurs plus élevées en hémicellulose et cellulose chez le matériel âgé (Monteuuis et Genestier 1989).

322. Profils méristématiques

Les différences de morphologie foliaire entre les matériel juvénile et âgé chez *Sequoiadendron giganteum* nous ont incités à examiner l'aspect des méristèmes primaires caulinaires, à l'origine de la phyllogenèse et de la phyllotaxie (Monteuuis 1987b). Les observations en microscopie optique ont permis d'établir que le contour des dômes méristématiques de cette espèce s'assimile à une régression curvilinéaire de la forme $y = ax^b$. Ces profils méristématiques sont significativement influencés par l'âge des sujets d'origine et le stade végétatif des bourgeons d'où sont issus les méristèmes. Par rapport aux observations correspondant au repos végétatif, les méristèmes prélevés lors du débourrement présentent une plus grande analogie morphologique avec le type juvénile évasé caractéristique, par contraste avec le type mature plus oblong. Ces différences correspondent aux changements de morphologie foliaire plus ou moins perceptibles lors de la reprise de croissance.

323. Mesures intra-méristématiques

Les investigations microscopiques ont été approfondies au niveau intra-méristématique sur le même matériel expérimental (Monteuuis 1989c). Les mesures cytomorphologiques réalisées à partir des coupes longitudinales médianes des points végétatifs ont permis de confirmer, à travers les valeurs du rapport diamètre/hauteur notamment, que les méristèmes de sujets juvéniles sont plus évasés, plus volumineux et contiennent plus de cellules que les méristèmes d'individus âgés, indépendamment du fait que le volume cellulaire varie en fonction de l'âge et du stage végétatif du matériel végétal d'origine.

Plus généralement, l'influence de l'état végétatif s'exerce de façon très significative pour chacun des critères précédents. Le débourrement correspond à une augmentation très nette des valeurs obtenues durant le repos végétatif, qui avoisinent alors les résultats caractérisant le matériel jeune. C'est notamment le cas pour le rapport nucléoplasmique, plus élevé pour les sujets juvéniles, surtout en période de repos végétatif. La différence avec le matériel âgé a tendance à s'estomper lors du débourrement, et n'est pas significative en ce qui concerne l'activité mitotique, stimulée par la reprise de croissance (Monteuuis 1989c).

Les valeurs élevées demeurent malgré tout l'apanage du matériel juvénile. Ceci est pleinement vérifié en ce qui concerne le volume du dôme méristématique et son effectif cellulaire, où les différences entre matériel jeune et âgé persistent de façon très marquée, que ce soit durant le repos végétatif ou le débourrement (Monteuuis 1989c).

33. APPROCHE BIOCHIMIQUE

331. Composés phénoliques et peroxydases

Les analyses biochimiques ont principalement porté sur *Sequoiadendron giganteum*. Dans le cadre d'études de microbouturage de cette espèce (Monteuuis et Bon 1986), nous avons pu établir une relation entre la teneur en composés phénoliques solubles et le degré de ramification des axes, différente selon qu'il s'agit de matériel juvénile ou âgé. Pour les sujets juvéniles, systèmes architecturaux à ramifications bien hiérarchisées, les dosages effectués montrent un gradient croissant basipète de la teneur en polyphénols dans les extrémités d'axe d'ordre 1, qui n'existe pas au niveau des ramifications d'ordre 2. Chez le clone mature, ce gradient est croissant acropète pour les axes d'ordre 1 et d'ordre 2. Ces résultats mériteraient d'être approfondis en relation avec les approches architecturales, d'autant que les teneurs initiales en polyphénols des explants seraient susceptibles d'influer sur leurs capacités organogènes ultérieures en conditions de culture *in vitro*. Les analyses effectuées sur des microboutures de *Sequoiadendron giganteum* relativement à leur aptitude à l'allongement, puis la néoformation de racines *in vitro* abondent dans ce sens. Les dosages peuvent varier considérablement, en liaison avec les manifestations organogènes observées *in vitro*, rhizogenèse adventive notamment, en fonction des échantillons et au cours du temps au sein d'un même clone. Ces variations importantes et intempestives nous ont amenés à considérer les dosages de composés phénoliques totaux plus comme des indicateurs d'état physiologique que des marqueurs fiables de l'âge physiologique, moins fluctuants en fonction du temps et des conditions environnementales que l'état physiologique, du moins en ce qui concerne *Sequoiadendron giganteum* (Monteuuis et Bon 1986, Monteuuis et al 1987a).

332. Nucléotides et acides nucléiques

Les analyses biochimiques se sont poursuivies sur le même matériel expérimental, mais au niveau des extrémités végétatives caulinaires eu égard au rôle déterminant des méristèmes primaires dans les phénomènes de changement de phase (Monteuuis 1989a et b). Les dosages afférents révèlent que la reprise de croissance correspond à une nette augmentation de la concentration en adénosine tri-phosphates (ATP), en nucléosides tri-phosphates non-adényliques (NTP) et en acides ribonucléiques (ARN) dans les apex de clones juvénile et âgés de *Sequoiadendron giganteum*, tandis que les concentrations en acides désoxyribonucléiques (ADN) ne varient pas

significativement entre le repos végétatif et le débourrement (Monteuuis et Gendraud 1987). Les rapports des concentrations en ATP et NTP d'une part ([ATP]/[NTP]), ARN et ADN d'autre part ([ARN]/[ADN]) sont nettement plus élevés chez le jeune matériel que chez le matériel âgé durant le repos végétatif. Lors de la reprise de croissance, ces différences entre les deux classes d'âge s'estompent et ne demeurent significatives que pour les nucléosides triphosphates.

Ces dosages ont révélé de grosses fluctuations entre les divers échantillons d'un même clone, indiquant d'importantes fluctuations d'état physiologique au niveau des zones apicales, et justifiant la méthodologie expérimentale adoptée (Monteuuis et Gendraud 1987).

La nette augmentation des teneurs en ARN au niveau des méristèmes primaires caulinaires lors de la reprise de croissance a pu être confirmée par des dosages cytophotométriques sur le même matériel expérimental, sans différence sensible en fonction de l'âge (Monteuuis 1989c).

333. Protéines

A l'instar des composés phénoliques, ces autres effecteurs du métabolisme auxinique que sont les peroxydases montrent de trop grosses variations dans le temps pour pouvoir être considérées comme des marqueurs fiables de l'âge physiologique, du moins chez *Sequoiadendron giganteum*. Elles paraissent en revanche plus appropriées en tant qu'indicateurs d'état physiologique, éminemment variable. C'est du moins ce qu'il ressort de l'étude de profils peroxydasiques en relation avec les capacités organogéniques *in vitro* de microboutures de cette même espèce (Monteuuis et al 1987a). Le distinguo est important. Ces aspects ont été développés dans plusieurs publications (Monteuuis et Bon 1986, Monteuuis et al 1987a, Monteuuis 1988, 1989a et b).

La possibilité de disposer, toujours chez *Sequoiadendron giganteum*, d'un échantillonnage relativement riche de génotypes juvéniles et âgés (Monteuuis 1985) a permis de contrecarrer les effets génotypiques perturbateurs dans la recherche de marqueurs protéiques de l'âge physiologique et de l'aptitude au clonage conforme associée. Ces travaux ont débouché sur la mise en évidence d'un polypeptide d'origine membranaire de 16kDa (Bon 1988b). Ce marqueur fiable et relativement peu influencé par les variations intempestives d'état physiologique a permis de confirmer le caractère endogène du rajeunissement obtenu par culture de méristèmes. "J16" fait défault dans le clone centenaire, mais est bien présente dans la lignée "mériclonale" rajeunie issue du même clone centenaire, comme dans le clone juvénile témoin (Bon et Monteuuis 1991).

Conjointement, la mise au point de techniques d'électrophorèses bidimensionnelles à partir de structures végétatives très réduites allant jusqu'à

un seul méristème a permis de comparer les populations protéiques entre les formes matures et juvéniles, ou rajeunies, d'un même génotype. Là encore, le fait de s'intéresser aux phénomènes de changement de phases (Monteuuis et Bon 1990) au sein d'un même génotype permet d'éviter les influences génotypiques perturbatrices. Le vieillissement correspondrait à une diminution à la fois sur le plan quantitatif et qualitatif des populations protéiques des extrémités végétatives apicales. C'est du moins ce qu'il ressort des observations effectuées sur des lignées topoclonales à caractère mature et juvénile de *Sequoia sempervirens* d'une part (Bon et al 1994), et d'une lignée mériclonale rajeunie de *Sequoiadendron giganteum* comparée au clone mature originel d'autre part (Bon et Monteuuis 1991).

Cette approche analytique appliquée à des microgreffes de la même espèce révèle que si les greffons à morphologiquement rajeunis présentent incontestablement des similitudes avec le matériel juvénile ou rajeuni, leurs homologues à morphologie mature caractérisée provenant du même clone âgé se distinguent par une population protéique globalement plus dense avec une intensification de la présence de protéines acides (Bon et Monteuuis 1987). Le fait que des greffons temporairement rajeunis du point de vue morphologique à l'issue du microgreffage révèlent par "westernblot" un deuxième polypeptide de masse moléculaire légèrement inférieure à "J16" (Bon 1988b), suggère une influence du porte-greffe, entre autres, au niveau des protéines du greffon. Le microgreffage pourrait par exemple permettre le passage de peptides du porte-greffe vers le greffon. Cette hypothèse ressort également des investigations de Huang et al (1992) sur la même thématique dans le cadre du microgreffage de *Sequoia sempervirens*.

34. COMMENTAIRES

La caractérisation de l'aptitude à la reproduction végétative vise à progresser dans la compréhension des mécanismes physiologiques sollicités, pour une meilleure efficacité, d'un point de vue pratique, des techniques de propagation asexuée. L'approche biochimique parait à cet égard plus prometteuse en vue de déterminer l'origine endogène des indices observés *de visu* et *a posteriori.* Les travaux entrepris dans cette voie ont été considérablement perturbés par les fluctuations des dosages effectués au cours du temps, au sein d'échantillons à potentialités organogéniques bien différentes, en fonction de la nature des molécules considérées. Cette variabilité dans le temps, qui confirme les observations *de visu* et atteste le bien-fondé des méthodes d'investigation multi-disciplinaires, ne saurait être sous-estimée lors de la recherche de marqueurs fiables de l'aptitude à la multiplication végétative, sous peine de conclusions erronées. Il a paru justifié d'insister fortement sur ce point (Monteuuis et Bon 1986, Monteuuis et al 1987a, Monteuuis 1988, 1989a et b).

4. DISCUSSION GÉNÉRALE:

VIEILLISSEMENT ET MULTIPLICATION VÉGÉTATIVE

Les travaux présentés confirment l'influence globalement négative du phénomène de vieillissement sur la "compétence à la multiplication végétative" (Nozeran 1978) des espèces arborescentes.

Le sujet traité permet d'aborder cette problématique à deux niveaux, à savoir à l'échelle de l'individu, puis à l'échelle du clone.

41. VIEILLISSEMENT ET MULTIPLICATION VÉGÉTATIVE À L'ÉCHELLE DE L'INDIVIDU

En référence à Fortanier et Jonkers (1976), il est d'usage de distinguer le vieillissement chronologique, le vieillissement ontogénétique et le vieillissement physiologique. La pertinence de ces concepts par rapport à l'aptitude à la reproduction végétative à l'échelle de l'individu, déjà argumentée de manière approfondie à partir d'observations concrètes (Monteuuis 1988, 1989a et b, Monteuuis et Bon 1998), mérite d'être discutée de nouveau.

411. Vieillissement chronologique

L'âge chronologique pour un individu issu de semis se définit à travers l'intervalle de temps écoulé depuis la germination jusqu'à l'instant présent (Fortanier et Jonkers 1976). Cette référence a été utilisée pour renseigner globalement pour une espèce sur la durée de la phase juvénile, qui s'achève classiquement par l'apparition des organes reproducteurs (Wareing 1959, Doorenbos 1965, Hackett 1985).

La principale lacune déjà dénoncée (Monteuuis 1988) de cette référence à l'échelle de l'arbre est de ne pas prendre en compte l'existence de gradients intra-individu de degré de juvénilité, d'autant plus marqués que le sujet de référence est développé. Cette hétérogénéité ou "parcellisation" (Nozeran 1978) physiologique s'illustre par exemple à travers les différences d'aptitude à la néoformation de racines en fonction de la proximité de l'appareil racinaire constatées par de nombreux auteurs (Passecker 1947, Doorenbos 1965, Borchert 1976, Bonga 1982). L'influence de la position de la bouture *in situ* sur son enracinement et son développement ultérieur ressort de nos travaux de bouturage pré-cités, et a fait récemment l'objet d'une synthèse (Monteuuis 1998). Ce gradient de capacités à la reproduction végétative est à l'origine de la notion de "topoclones" (Franclet 1981) particulièrement adaptée à *Sequoia sempervirens* (Bon et al 1994).

412. Vieillissement ontogénétique

L'âge, et par suite le vieillissement ontogénétique, se définit par rapport au développement spatial de l'individu au cours de l'ontogenèse, assuré par :l'activité du méristème primaire caulinaire édificateur. En conditions naturelles, le vieillissement ontogénétique repose sur la dualité entre le temps et le développement spatial dans la mesure où, en conditions naturelles, un certain stade ontogénétique ne sera atteint qu'au bout d'un certain laps de temps. Le fait que les organes dernièrement formés à la périphérie du houppier soient considérés comme les plus jeunes du point de vue de l'âge chronologique et comme les plus vieux sous l'aspect de l'âge ontogénétique (Hackett 1985) traduit cette dualité, sur laquelle insistent plus récemment Lawson et Poethig (1995) en employant la terminologie consacrée de "developmental time".

L'expression du vieillissement ontogénétique est particulièrement flagrante chez les espèces hétéroblastiques telles que *Acacia mangium, Eucalyptus tenuiramis* (Wiltshire et Reid 1992), ou à dimorphisme foliaire contrasté entre les phases juvéniles et matures, en reconnaissant à la morphologie foliaire sa valeur d'indice de degré de maturité (Schaffalitzky de Muckadell 1959, Doorenbos 1965). Conjointement, il parait tout à fait logique et pertinent de

considérer les méristèmes primaires édificateurs et générateurs du mouvement morphogénétique (Nozeran 1980) comme le siège du vieillissement ontogénétique. Cet aspect a été particulièrement développé par Schaffalitzky de Muckadell (1959). Robinson et Wareing (1969), puis réactualisé par Sussex (1989) notamment. Fortanier et Jonkers (1976) quant à eux proposent de concevoir le vieillissement ontogénétique en nombre de mitoses produites au niveau méristématique.

Il nous a paru important de se focaliser sur ce point, en insistant sur le fait que les différents axes et méristèmes d'un arbre proviennent de l'activité édificatrice du seul et même méristème originel situé initialement au pôle apical de l'embryon (Monteuuis 1989b, Monteuuis et Bon 1998). C'est ce méristème originel qui a donné naissance au cours du développement à un nombre croissant de méristèmes "fils", d'âges ontogénétiques de plus en plus élevés, comme illustré Figure 3. Ces méristèmes "fils" ont pu demeurer latents au sein de bourgeons proventifs, ou participer à l'édification du complexe architectural.

Les diverses manipulations de pieds-mères telles que tailles ou recépage, visent en fait à stimuler la production d'axes plus aptes à la reproduction végétative, à partir de méristèmes d'autant plus jeunes d'un point de vue ontogénétique qu'ils sont situés prés du pôle racinaire. Ainsi que nous l'avons déjà souligné (Monteuuis et al 1990), il s'agit en fait de l'exploitation à des fins de propagation végétative des phénomènes de réitérations de type traumatique décrits par ailleurs (Edelin 1977, Barthélémy 1990, Crabbé 1990). De telles pratiques retardent artificiellement le vieillissement ontogénétique, en dépit de l'âge chronologique croissant de la plante (Monteuuis 1988, 1989b). Et les observations concrètes confèrent en la matière plus d'importance à l'âge ontogénétique qu'à l'âge chronologique (Monteuuis 1988, 1989a et b).

Tout ceci ne saurait occulter les cas de déviations ou rajeunissements morphologiques particulièrement visibles chez les espèces hétéroblastiques, et observés, ne serait-ce que de façon fugace, en zones tempérées (Monteuuis 1989b) et tropicales (Barthélémy 1990). La prise en considération de ces observations bien réelles, et qui, en tant que telles, ne sauraient être ignorées, nous ont incités à prendre un certain recul par rapport à la vision de Fortanier et Jonkers (1976) du vieillissement ontogénétique génétiquement programmé au sein des méristèmes. En effet, comment alors interpréter ces "retours en arrière" (Nozeran et al 1982)?

413. Vieillissement physiologique

La notion d'âge physiologique reprise et développée principalement par Borchert (1976) parait plus réaliste et séduit par sa cohérence, sa pertinence. Elle permet d'interpréter bon nombre d'observations morphologiques,

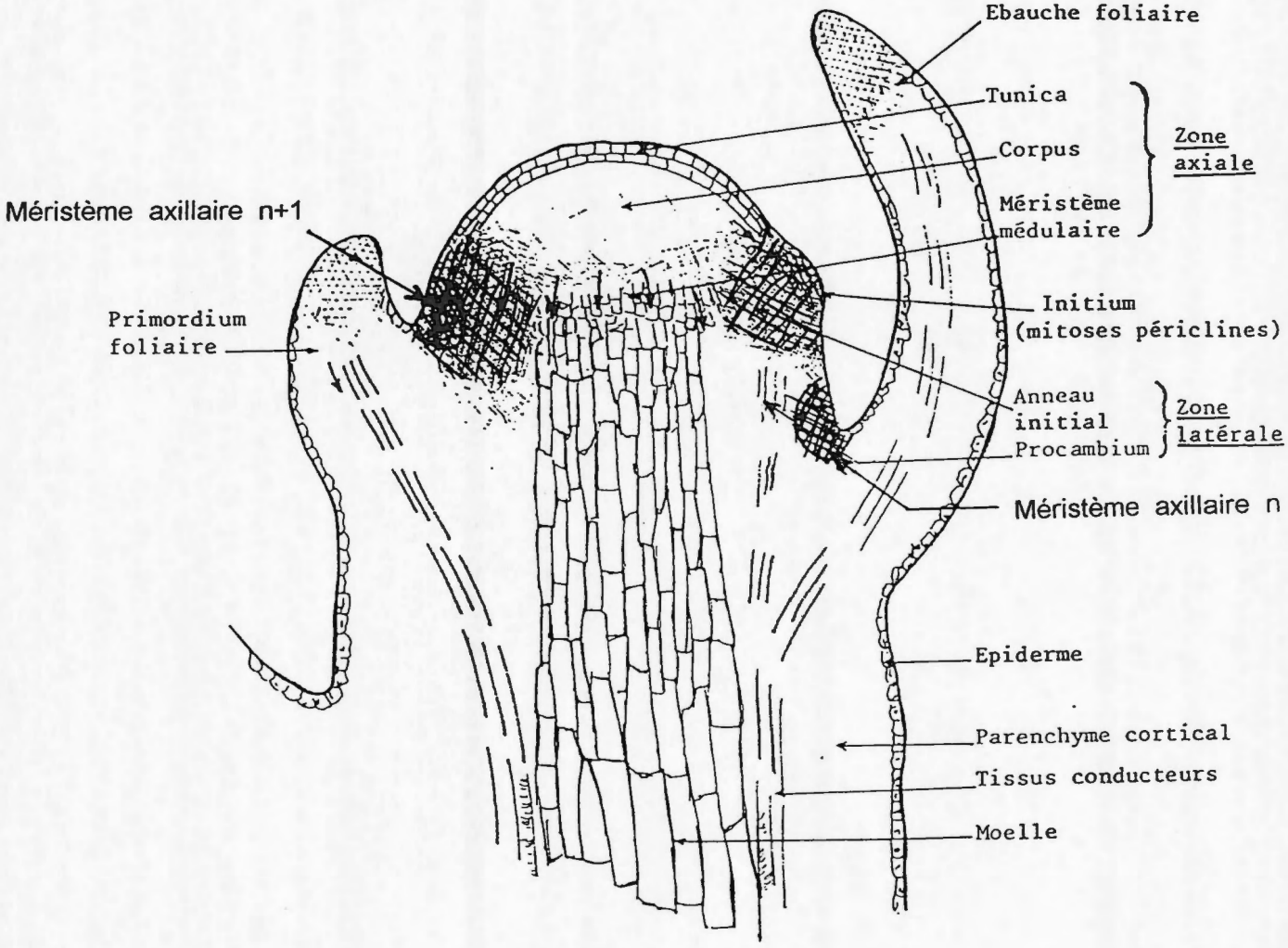

Figure 3. Représentation schématique de l'organisation histo-cytologique d'un méristème primaire caulinaire, vu en coupe. Les zones hachurées sont le siège d'une intense activité mitotique qui, à travers des divisions périclines et anticlines, assure l'organogenèse, notamment la formation des feuilles et en zone péri-axiale, des méristèmes "fils" à l'origine de la formation des rameaux axillaires. Sur le plan de l'âge ontogénétique, le méristème axillaire "n" est moins vieux que son homologue "n+1", lui-même plus jeune que le méristème terminal originel en position sommitale (d'après Monteuuis et Bon 1998).

organogénétiques, physiologiques, histo-cytologiques et biochimiques, comme nous l'avons montré sur *Sequoiadendron giganteum* (Bon 1988a et c, Monteuuis et Bon 1986, Monteuuis 1987, Monteuuis et Gendraud 1987, Monteuuis et al 1987a, Monteuuis 1988, 1989b).

Ainsi, conformément à la conception de Krenke (1940) reprise par Franclet (1983), les potentialités juvéniles initiales deviendraient, au cours du développement ontogénétique de l'arbre en fonction du temps, de plus en plus restreintes dans l'espace, présentant le maximum d'intensité au moment du débourrement dans les extrémités apicales des tiges, comme illustré Figure 4a.

Cette interprétation, établie principalement à partir d'observations de la morphologie foliaire, s'inspire directement de la notion de cyclophysie au sens de Schaffaliztky de Muckadell (1959). La différence fondamentale par rapport au concept de vieillissement ontogénétique graduel et irréversible proposé par Passecker (1947), et schématisé Figure 4b, réside en l'existence de foyers virtuels de juvénilité au sein des méristèmes primaires caulinaires, même si ces "créneaux" ou "fenêtres" de potentialités juvéniles deviennent de plus en plus réduites d'un point de vue spatio-temporel au cours de la croissance. Ils nous a paru fondé de développer ces différentes notions à plusieurs occasions (Monteuuis 1988, 1989a et b), en les explicitant par des schémas (voir Figures 5 et 6).

Cette conception du vieillissement physiologique des méristèmes conformément à la vision de Borchert (1976) permet d'interpréter également les phénomènes de "retour en arrière" ou de rajeunissement. Il s'agirait plus vraisemblablement de l'expression de massifs cellulaires restés juvéniles au sein des méristèmes - sous l'effet de systèmes corrélatifs inhibiteurs (Nozeran 1978) - , que d'un rajeunissement véritable de cellules matures. La restriction dans l'espace, et principalement dans le temps, de ces "créneaux" de juvénilité *in situ* permettrait d'expliquer la probabilité, d'autant plus faible que l'individu de référence est développé, de rajeunir radicalement par culture de méristème excisé. En vertu de ces arguments, la miniaturisation des explants peut être effectivement considérée, comme le prétend Nozeran (1978, 1985, 1986), comme "une condition nécessaire mais non suffisante à un retour en arrière" (Monteuuis 1988, Monteuuis et Bon 1998).

42. VIEILLISSEMENT ET MULTIPLICATION VÉGÉTATIVE À L'ÉCHELLE DU CLONE

L'exemple de certaines espèces de *Thuja* du nord du continent américain, *Thuja plicata* notamment, qui se propagent naturellement par marcottage depuis des siècles (Edelin 1997, communication personnelle), illustre le maintien des capacités ontogénétiques des méristèmes au cours du temps, indépendamment d'un âge ontogénétique croissant. Il convient en effet de réaliser que la

4a: Concept de Passecker (1947) attaché au caractère irréversible du processus de vieillissement en fonction du développement ontogénétique.

4b: Concept de Krenke (1940), repris par Franclet (1983) du vieillissement selon un mode séquentiel réitéré en fonction de l'allongement des unités de croissance successive au cours du développement ontogénétique.

Figures 4. Représentation schématique des concepts de Passecker (1947) et de Krenke (1940), interprété par Franclet (1983, du vieillissement des espèces arborescentes en fonction du développement ontogénétique de l'individu (tiré de Monteuuis et Bon 1998).

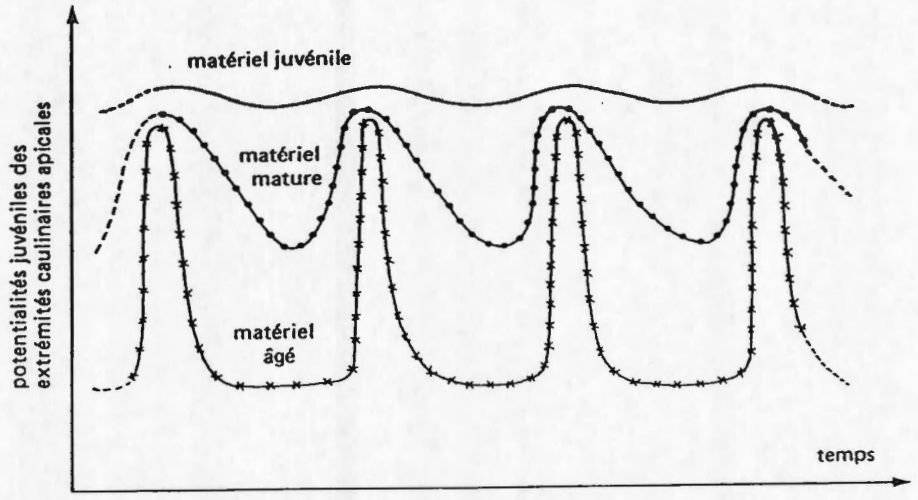

Figure 5. Interprétation schématique et hypothétique des profils évolutifs, en fonction du temps, des potentialités juvéniles d'extrémités apicales caulinaires dans le cas de matériels juvéniles, matures et âgés, définis par rapport à leur âge physiologique. Les variations seraient imputables aux fluctuations d'activité de croissance, les maxima correspondant au stade pré-élongation (notion de cyclophysie) et leur fréquence pouvant varier en fonction des matériels (cas de polycyclisme) (tiré de Monteuuis 1989a et b).

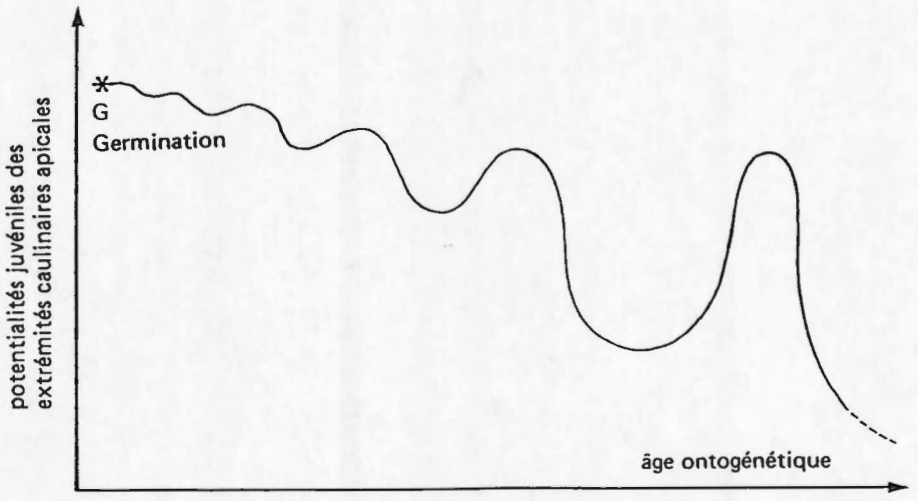

Figure 6. Interprétation schématique du profil évolutif des potentialités juvéniles au sein des extrémités apicales en fonction de leur âge ontogénétique. Il semble en effet légitime de prétendre que ces potentialités juvéniles sont étroitement dépendantes de l'âge physiologique, et diminuent lorsque ce dernier augmente (voir texte) (tiré de Monteuuis 1989a et b)

propagation clonale est assurée par des méristèmes dont l'origine cellulaire remonte à la formation du méristème initial situé au pôle apical de l'embryon. Le clonage revient donc à perpétuer théoriquement à l'infini la filiation organogénique évoquée précédemment à l'échelle de l'individu entre le méristème originel et les méristèmes "fils" situés à l'extrémité des axes qu'ils ont édifiés.

La propagation par greffage de cultivars fruitiers s'accompagne à chaque nouvelle génération d'une récapitulation du phénomène de changement de phases, se traduisant pratiquement par un délai de mise à fruit correspondant à la période juvénile. Ce processus se perpétue avec une régularité bien établie pour certaines variétés depuis des siècles, comme le rappelle Sax (1962).

Il en est de même pour les espèces ligneuses ornementales et forestières, propagées par greffage, marcottage ou bouturage. Les clones hybrides de peupliers illustrent les potentialités ontogénétiques des méristèmes garant d'une reproduction clonale conforme au fil des générations de bouturage..

Ces observations, illustrées sous la forme d'un schéma synthétique Figure 7, méritent d'être considérées dans toute leur signification par rapport aux phénomènes de vieillissement classiquement admis. Elles constituent un argument majeur semble-t-il à l'encontre du vieillissement irréversible génétiquement programmé au sein de méristèmes et conduisant inéluctablement à la mort de l'individu et du génotype.

La propagation végétative apparaît à cette enseigne comme un élément méthodologique inestimable pour discerner la pertinence du vieillissement irréversible génétiquement programmé, par rapport à celle du vieillissement physiologique réversible, réfutant des points de vue profondément figés en la matière (Irish et Nelson 1988, Poethig 1988, 1990, Lawson et Poethig 1995).

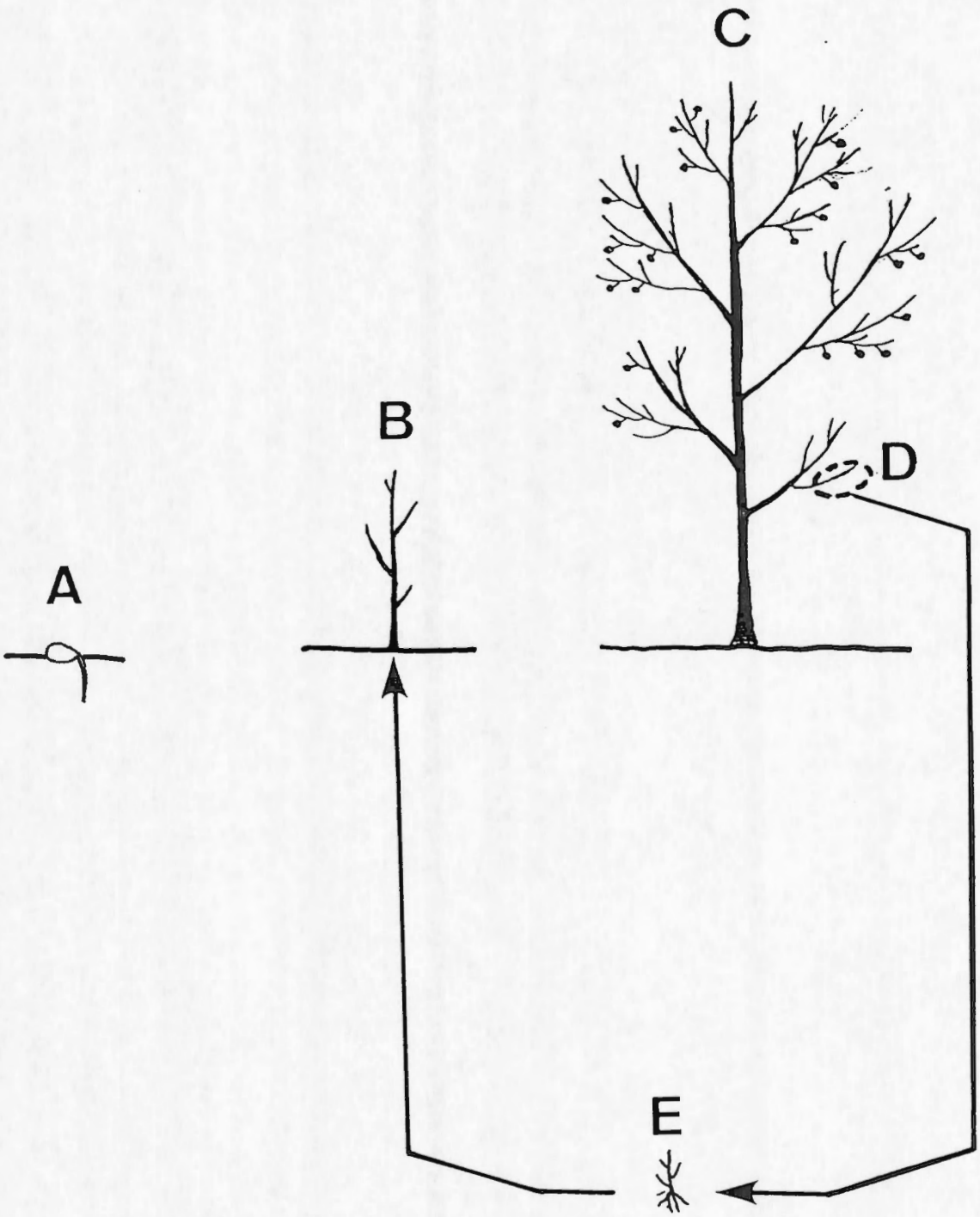

Figure 7. Développement ontogénétique et propagation clonale chez les espèces arborescentes. A) Germination. B) Phase juvénile (jusqu'à l'apparition des organes reproducteurs). C) Phase mature (florifère). D) Prélèvement d'un fragment d'axe végétatif dont l'origine cellulaire remonte au pôle apical de l'embryon. E) Cycle de multiplication végétative par bouturage ou greffage, qui peut être répété afin de perpétuer le clonage conforme, parfois pendant plusieurs siècles. On notera que chaque nouveau cycle débute par la phase juvénile, de durée relativement constante, phénomène bien connu en arboriculture fruitière.

5. CONCLUSION, PERSPECTIVES

Les travaux exposés ont été réalisés dans un esprit de Recherche axée sur le Développement, conformément aux objectifs professionnels assignés.

Ce contexte a permis d'appréhender les divers aspects de la multiplication végétative d'espèces arborescentes dans des situations très variées, aussi bien du point de vue climatique, de l'équipement et des installations, que du matériel végétal étudié. Les activités de terrain ou de pépinière ont favorisé une meilleure connaissance des espèces et de leurs particularités spécifiques en conditions naturelles, incitant à relativiser la notion de "plante modèle". La finalité appliquée des recherches entreprises a été le fil conducteur souhaitable pour éviter les risques de dispersion, particulièrement lors des investigations pluridisciplinaires.

L'étude du clonage de *Sequoiadendron giganteum* peut être objectivement considérée comme un exemple réussi de Recherche dans un esprit de Développement.

A partir d'une problématique concrète et d'un objectif appliqué clairement défini, la méthodologie expérimentale, résolument basée sur le pragmatisme et l'empirisme, a progressé logiquement, sur la voie de la miniaturisation, de la finesse et de la précision pour une meilleure pertinence des résultats.

La collaboration au niveau institutionnel des organismes de Recherche appliquée, voire de Développement, et de l'Université a permis une approche multidisciplinaire et synergique de la problématique. qui s'est valorisée à travers un certain nombre d'acquis. Ceux-ci ont été attestés au niveau scientifique par des publications internationales et au niveau pratique par l'obtention d'une lignée mériclonale véritablement rajeunie.

Nous ne pouvons qu'adhérer à cette conception de la Recherche qui concilie les intérêts de la Science et du Développement.

L'aptitude à la multiplication végétative, et plus précisément au clonage conforme, n'est qu'un aspect appliqué de l'incidence des phénomènes de changement de phases. Ceux-ci influent également sur d'autres manifestations telles que les délais de mise à fleurs et de fructification... A des degrés variables en fonction des espèces, bon nombre de systèmes de production centrés sur l'utilisation de l'Arbre, sous diverses formes, que ce soit en foresterie ou agro-foresterie, en arboriculture fruitière, horticulture ornementale, hévéa-culture,... sont effectivement concernés. Il parait inutile d'insister davantage sur l'intérêt appliqué de cette problématique, suffisamment développé dans la bibliographie. Un récent examen en la matière révèle que cette thématique du changement de phases suscite toujours de l'intérêt auprès des chercheurs avec, comme on pouvait l'espérer, la mise en oeuvre des techniques de biologie moléculaire afin de cerner son déterminisme, sa causalité. Si les efforts consentis dans de ce domaine ne peuvent qu'être loués, il est toutefois nécessaire de garder suffisamment de discernement et de recul vis à vis de cette problématique complexe, et de certaines conceptions exclusivement "molécularistes" de la question (Poethig 1988, Greenwood et Hutchinson 1993, Greenwood 1995, Mellerowicz et al 1995). La possibilité de mener des recherches au niveau de l'ADN ne saurait occulter la pertinence de certains concepts et de bon nombre d'observations qui, bien qu'émanant de moyens analytiques moins élaborés, n'en demeurent pas moins significatives du point de vue physiologique.

La prise en considération de l'ensemble de ces éléments incitent à poursuivre sur cette thématique de recherche, en s'inspirant du travail mené sur *Sequoiadendron giganteum*, crédité par un recul de dix années.

Ainsi, l'importance des méristèmes primaires caulinaires relativement au phénomène de changement de phase ne saurait effectivement être remise en question. L'approfondissement des connaissances au niveau méristèmatique nécessite la mise au point de techniques d'investigation adaptées. Nous avons privilégié ces aspects, tant du point de vue de la culture *in vitro* que des méthodes analytiques, et plus particulièrement Bon (1988b et c, 1989) dans le domaine des indices biochimiques. Il parait encore plus fondé qu'il y a dix ans de porter beaucoup d'espoirs aux techniques d'immunolocalisation appliquées aux structures méristématiques afin de circonscrire les zones à hautes potentialités juvéniles. En conditions non-dénaturantes, ces massifs tissulaires pourraient être excisés à l'aide de techniques de microchirurgie par exemple, pour être introduits en culture *in vitro* en vue du clonage. Ceci supose une approche multi-disciplinaire du problème, où la biochimie et la biologie moléculaire doivent occuper une place essentielle, notamment pour la mise en évidence de marqueurs de l'âge physiologique nécessaires pour les techniques d'immunolocalisation. A cette enseigne la mise en évidence par Bon (1988b) de "J16", polypeptide indiquant le degré de juvénilité chez *Sequoiadendron giganteum*, fait figure de précurseur, comme en témoignent les travaux de Besford et al (1996) notamment sur *Prunus avium*. L'intérêt de tels marqueurs est évident sur le plan pratique, dans le cadre de tests clonaux par exemple,

afin de quantifier et d'homogénéiser le degré de rajeunissement des clones susceptible de fausser l'appréciation des valeurs génotypiques.

D'un point de vue plus fondamental, ces marqueurs pourraient permettre de progresser dans la compréhension au niveau moléculaire des phénomènes de changement de phase, et de faire enfin la part des choses entre leur origine génétique ou épigénétique, cette dernière hypothèse paraissant objectivement encore la plus vraisemblable à ce jour (Monteuuis 1988, 1989a et b, Huang et al 1995).

Terminologie

La thématique traitée fait appel à une terminologie quelque peu spécialisée, surtout dans le domaine du changement de phase, abondamment traité en langue anglaise et d'où certains mots peuvent découler directement.

La liste non-exhaustive de définitions proposée ci-dessous a pour but de clarifier quelques uns de ces termes, pour certains déjà explicités dans le texte, afin de limiter les confusions, les ambiguïtés voire les risques d'ésotérisme (voir aussi Monteuuis 1988, Monteuuis et Bon 1998). Le plus souvent, nous nous sommes efforcés d'employer les termes conformément à leur définition courante stipulée dans les dictionnaires de langue française.

Age, âgé, jeune, vieux: définis classiquement, par rapport à la référence temporelle, en fonction du laps de temps écoulé.

Age chronologique: défini, à l'échelle de l'individu, par "le temps écoulé depuis la germination de la graine " (Fortanier et Jonkers 1976), et par extension, par la durée d'existence de la structure de référence. Synonyme d'âge; l'adjectif "chronologique" est précisé pour marquer le distinguo avec l'âge physiologique et l'âge ontogénétique.

Age ontogénétique: exprime le "passé morphogénétique", qui peut se traduire selon Fortanier et Jonkers (1976) par la quantité présumée de mitoses au sein des méristèmes édificateurs, en fonction de leur implication dans le déroulement des séquences ontogénétiques.

Age physiologique: essentiellement sous déterminisme physiologique, et appréhendé à travers différentes manifestations révélatrices telles que l'aptitude à la floraison, à la rhizogenèse adventive....(Borchert 1976).

Arbre ou individu "Plus": présente des caractéristiques phénotypiques supérieures à celles de ses voisins susceptibles d'être dupliquées par reproduction végétative ou asexuée; se distingue de l'arbre ou individu "d'élite" dont la supériorité doit être établie sur base de sa descendance produite par voie sexuée.

Bourgeon proventif: bourgeon formé depuis longtemps, mais resté à l'état latent jusqu'à son "réveil organogénique".

Changement de phases: passage de l'état juvénile à l'état mature, voire sénescent, et inversement. Les "phases" sont essentiellement définies par rapport à des critères physiologiques, en relation avec la notion d'âge physiologique.

Clone: ensemble d'individus physiquement autonomes et indépendants génétiquement identiques à la "tête de clone" originelle, ou "ortet".

Clonage conforme: obtention d'un clone constitué de représentants conformes du point de vue phénotypique à la tête de clone originelle.

Cycloclone: sous-ensemble intra-clonal obtenu à partir d'un méristème à un certain stade de la cyclophysie (Durzan 1984).

Cyclophysie: traduit l'état de maturité physiologique d'un méristème donné à un instant donné, susceptible de varier de façon réitérée et cyclique, conformément au concept de Krenke (1940).

Hétéroblastique: exprime des différences très contrastées de morphologie au cours du développement ontogénétique.

Juvénile, juvénilité: synonyme de jeune; dans le contexte du changement de phases, l'état juvénile précède l'état mature et est caractérisé par l'absence d'organes reproducteurs, et généralement une grande aptitude à la rhizogenèse adventive et au clonage conforme.

Mature, maturité: classiquement et strictement caractérisé par l'apparition des organes reproducteurs.

Macropropagation: propagation végétative en conditions horticoles.

Mériclone: lignée intra-clonale régénérée à partir de la culture *in vitro* d'un seul méristéme.

Méristème: synonyme dans le texte de point végétatif ou méristème primaire caulinaire.

Micropropagation: propagation végétative en conditions de culture *in vitro*.

Mobilisation: obtention de la première génération de copies génétiques indépendantes et autonomes, induisant généralement une réactivation physiologique du matériel mobilisé.

Morphogenèse: évolution morphologique considérée sous divers aspects: caulogenèse, rhizogenèse, callogenèse, phénomènes de croissance,... La morphogenèse inclue l'organogenèse.

Multiplication végétative: mode de division asexuée permettant l'obtention de copies génétiques indépendantes et autonomes, sans préjuger de leur conformité phénotypique.

Néoténie: apparition inoppinée et anormale d'organes reproducteurs à partir de sujets jeunes.

Ontogenèse: édification de l'architecture de l'individu au cours du processus de développement.

Organogenèse: formation d'organe(s).

Ortet: individu sur lequel sont prélevés des fragments végétatifs, ou "ramets", en vue de la multiplication végétative. L'ortet se différencie du pied-mère en ce sens qu'il ne subit aucun traitement particulier destiné à stimuler son aptitude au bouturage.

Phyllogenèse: formation de feuilles.

Plagiotrope: présentant un angle de déviation par rapport à la verticale. Une croissance diagétrope s'effectue à l'horizontale, cas extrême de plagiotropisme.

Pousse épicormique: pousse adventive sur tronc, voire branche de gros diamètre.

Propagation végétative: synonyme de multiplication végétative.

Ramet: voir ortet.

Rajeunissement: réapparition partielle ou totale de caractéristiques des formes de jeunesse, sans préjuger de la stabilité dans le temps de ces "retours en arrière" (Nozeran 1978); défini classiquement par rapport à un état de référence.

Régénération végétative: "aspect de la reproduction végétative; implique la séparation d'une partie de la plante-mère et la formation d'organes totalement nouveaux à partir du fragment isolé" (Bigot 1976).

Rejet: Pousse vigoureuse émise à proximité du tronc.

Reproduction végétative: "ensemble des modalités assurant la reconstitution ontogénétique intégrale d'un organisme sans intervention d'un processus sexué" (Bigot 1976).

Rhizogenèse: formation de racines, éventuellement adventives à partir de structures de tiges lorsque précisé; ne pas confondre avec croissance racinaire.

Tête de clone: individu de semis d'où le clone tire son origine.

Topophysie: influence de la position topologique du ramet au sein de l'ortet sur son développement ontogénétique ultérieur (Schaffalitzky de Muckadell 1959).

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VIII. ANNEXE:

PRINCIPALES PUBLICATIONS SCIENTIFIQUES INTERNATIONALES

PHYSIOLOGIE VÉGÉTALE. — *Microgreffage de points végétatifs de Sequoiadendron giganteum Buchholz séculaires sur de jeunes semis cultivés in vitro.* Note de **Olivier Monteuuis,** présentée par Roger Gautheret.

Une nouvelle technique de microgreffage permet de greffer avec succès, sur de jeunes semis cultivés *in vitro*, des méristèmes de *Sequoiadendron giganteum* prélevés sur de jeunes pousses en repos végétatif de sujets centenaires. 35 % des greffons évoluent rapidement en pousses feuillées comparables au type morphologique juvénile.

PLANT PHYSIOLOGY. — *In vitro* micrografting of *Sequoiadendron giganteum* meristems.

A new micrografting technique allows to graft with success meristems taken from rest shoots of 100 *year-old* Sequoiadendron giganteum *on* in vitro *young seedlings. 35% of the grafts provide rapidly leafy shoots which look like juvenile morphological type.*

INTRODUCTION. — Plusieurs travaux ([3], [5], [9]) mentionnent que le greffage, sur de jeunes porte-greffes, d'axes végétatifs prélevés sur des sujets âgés peut favoriser la réacquisition de caractéristiques morphogénétiques de type juvénile au niveau du greffon. Cette évolution est d'autant plus facile à obtenir que le greffon est le moins différencié sur le pied-mère d'origine et nécessite généralement plusieurs générations de greffes successives ([4], [8]). En ce sens, le fait de limiter la taille du greffon au point végétatif peut être avantageux. Sur notre matériel d'études, *Sequoiadendron giganteum* Buchholz, ce projet est difficilement réalisable en conditions horticoles traditionnelles [9]. Pour cette raison, une technique de microgreffage *in vitro* dans un environnement plus adapté car mieux contrôlé en limitant les risques de contaminations, a été mise au point selon les modalités décrites ci-dessous.

MATÉRIEL ET MÉTHODES. — 1. *Obtention des porte-greffes.* — Les graines, conservées en chambre froide (+1°C), sont immergées durant 24 h dans une solution titrant 2 g/l de bénomyl et additionnée de quelques gouttes de mouillant Tween 80 afin de prévenir les risques de contaminations assez fréquents par *Botrytis cinerea.* Après un trempage de 4 à 5 mn dans une solution d'éthanol à 50 %, puis dans du chlorure mercurique à 1‰, les graines sont abondamment rincées dans quatre bains d'eau distillée stérilisée, puis réparties aseptiquement dans des bocaux de culture de 0,5 l garnis au tiers de leur hauteur de vermiculite imbibée d'eau stérilisée. Ces récipients, hermétiquement fermés par une bande de parafilm, sont ensuite disposés en chambre de culture où des tubes fluorescents « Mazda Fluor lumière du jour de luxe » assurent une intensité lumineuse de 10 W/m² pendant 16 h; la température est fixée à 23±1°C. 2 semaines plus tard, les radicules apparaissent. Au stade cotylédons étalés — émergence de l'épicotyle, soit 4 à 5 semaines après la germination, les plantules sont repiquées individuellement dans des tubes de culture droits de 20 × 160 mm coiffés de façon non hermétique de capuchons en matière plastique transparente. Chaque tube contient une motte cylindrique de 25 × 30 mm en fibres de polypropylène imbibée de 5 ml de la solution minérale de Murashige et Skoog [10] additionnée de 30 g/l de saccharose. Ce support est destiné à faire corps avec la plantule grâce à un système racinaire vigoureux. Dès que l'épicotyle atteint 2 à 3 cm de hauteur, le greffage peut être effectué.

2. *Origine des greffons.* — Les greffons sont prélevés sur des rameaux de *Sequoiadendron giganteum* centenaires greffés et élevés en conteneurs dans une salle climatique où les conditions ambiantes sont similaires à celles de la chambre de culture *in vitro*, exceptée l'intensité lumineuse fixée à 17 W/m² pour une humidité relative de 75 %.

3. *Technique de greffage.* — Les manipulations sont effectuées en conditions aseptiques; le greffage est réalisé sous loupe binoculaire et la source lumineuse est transmise par fibres optiques. Le porte-greffe, solidaire de son support synthétique (*fig.* 1), est délicatement amené vers l'orifice du tube de culture pour ne sortir que la partie supérieure de l'axe épicotylé (*fig.* 2) qui est alors entaillé latéralement par une incision longitudinale de 1 à 2 mm de long réalisée à l'aide d'un éclat de lame de rasoir (*fig.* 3). Le greffon, prélevé par une dissection rapide, est constitué par le dôme apical du méristème caulinaire avec ses primordia et éventuellement une ébauche foliaire; la base est taillée en biseau et l'ensemble, hors tout, n'excède pas 0,4 mm. L'explant, posé

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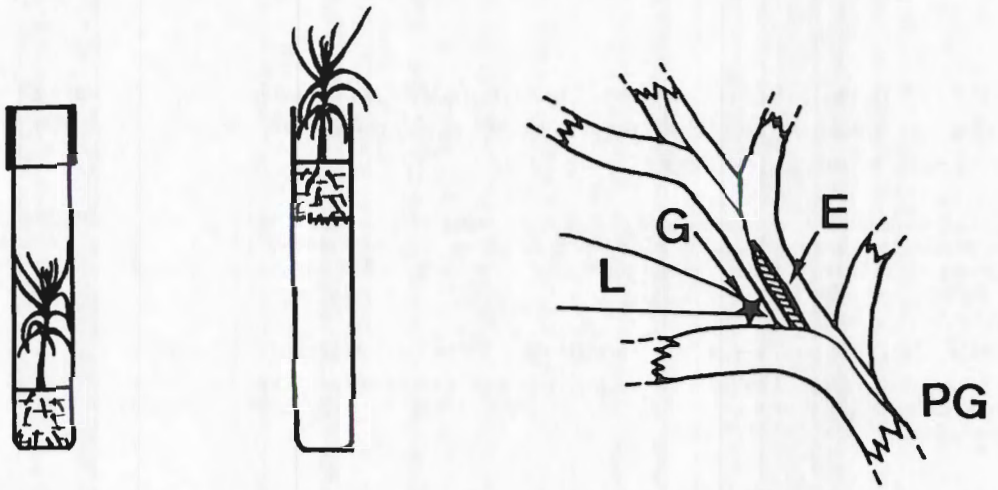

Étapes chronologiques du greffage. Fig. 1. : semis porte-greffe en culture *in vitro* sur motte synthétique. Fig. 2. : porte-greffe prêt à être greffé. Fig. 3. : le greffon (G) sur l'éclat de lame de rasoir ayant servi au prélèvement (L) est inséré dans l'entaille (E) effectuée sur l'axe épicotylé du porte-greffe (PG).

Micrografting successive stages. Fig. 1: young seedling stock-plant growing in vitro *on synthetic substrate. Fig. 2: stock-plant ready for grafting. Fig. 3: the scion (G) on the razor slide used for the removal (L) is introduced into the cut (E) made on the stock-plant epicotyl (PG).*

sur l'extrémité de l'éclat de lame de rasoir utilisé pour le prélèvement, est immédiatement et très délicatement inséré dans la fente préalablement effectuée sur le porte-greffe (*fig.* 3). Cette étape s'apparente, à une échelle beaucoup plus réduite, à la technique de greffage traditionnelle et il importe d'en respecter les modalités usuelles : rapidité, coupe franche, polarité du greffon, position correcte de l'implant afin de faciliter précocement l'adhérence entre greffon et porte-greffe. L'opération réalisée, le porte-greffe est replacé dans les conditions de culture initiales.

RÉSULTATS. — Le succès du greffage est conditionné par la dextérité du manipulateur et par l'état de la pousse au moment du prélèvement. En période de repos végétatif, nous obtenons 35 % de réussite sur 90 greffes réalisées. Au contraire, les méristèmes prélevés sur des axes en croissance ont tendance à s'oxyder, avant de dépérir. Dans les situations favorables, la soudure s'effectue rapidement par prolifération des tissus corticaux du porte-greffe. L'organogenèse, puis le développement de la nouvelle pousse commence 2 à 3 semaines après le greffage. Les toutes premières feuilles formées sont quelque peu atrophiées, conséquence vraisemblable du traumatisme causé par le greffage; puis la morphologie du greffon s'identifie au type juvénile [9].

Un sevrage progressif favorise la croissance de la nouvelle pousse en évitant une trop forte concurrence avec l'appareil caulinaire du porte-greffe. L'acclimatation en conditions horticoles est facilitée par la qualité du système racinaire développé sur substrat fibreux.

DISCUSSION, CONCLUSIONS. — La méthode proposée, applicable dans son principe à bon nombre d'autres espèces, permet de greffer avec un rendement de 35 % des points végétatifs de sujets âgés sur de jeunes semis cultivés *in vitro*.

Du point de vue technique, la motte en substrat synthétique est avantageuse car elle permet de sortir sans dommage le porte-greffe du tube pour faciliter le greffage. D'autre part, chimiquement neutre, elle garantit la composition de la solution nutritive qui peut être modifiée en cours de culture si besoin est. La fente latérale, quant à elle, évite le

dessèchement du méristème implanté et favorise l'union du greffon et du porte-greffe dans un micro-environnement propice, à l'abri d'une lumière trop forte.

Cette possibilité de greffer des méristèmes peut être exploitée à diverses fins, en biologie fondamentale comme dans des domaines plus appliqués tels que l'arboriculture fruitière ([1], [2], [6], [7]). C'est également un moyen de profiter des avantages reconnus de la culture des méristèmes en substituant au milieu de culture artificiel, généralement gélosé, un support naturel : le porte-greffe. Cette solution paraît plus judicieuse, tant du point de vue pratique que physiologique, notamment pour recouvrer certaines caractéristiques des formes juvéniles telles que l'aptitude à la multiplication végétative. Les premiers résultats d'une étude comparative sur le clonage d'arbres âgés à partir de méristèmes vont dans ce sens.

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Morphologie végétale/*Plant Morphology*

Profils méristématiques de séquoias géants (Sequoiadendron giganteum Buchholz) jeunes et âgés durant les stades de repos végétatif et de débourrement

Olivier MONTEUUIS

Résumé — Le contour des dômes méristématiques de séquoias géants (*Sequoiadendron giganteum* Buchholz) peut être décrit au moyen d'une régression curvilinéaire de la forme $y=ax^b$. Les profils méristématiques obtenus sont significativement influencés par l'âge des sujets et le stade végétatif des bourgeons d'où sont issus les méristèmes. Par rapport aux observations correspondant au repos végétatif, les méristèmes prélevés lors du débourrement présentent une plus grande analogie morphologique avec le type juvénile évasé caractéristique. Ces résultats sont exposés en relation avec l'aptitude à la multiplication végétative du matériel considéré.

Apical meristem outlines of young and mature giant sequoias (*Sequoiadendron giganteum* Buchholz) during rest phase and budbreak

Abstract — Apical meristem outlines of giant sequoias (Sequoiadendron giganteum *Buchholz) could be described through a curvilinear regression of the following form:* $y=ax^b$. *The profiles obtained were connected both to the age of the donor tree and to the phenology of the buds from which the apices were excised. Thus, when compared to the rest period, meristems removed during budbreak showed a larger basal surface, tending in this way to the juvenile characteristics. These results were considered in relation to the cloning capacities of the ortets.*

Abridged English Version — In the same way as for a lot of ligneous species [1], success of *in vitro* meristem cultures of giant sequoia (*Sequoiadendron giganteum*) depends on the age of the donor tree and on the time of the explant removal [2]. The influence of these two factors on the apical meristem conformation was analysed, referring to 18 month-old and 100 year-old giant sequoia [3] during rest phase and budbreak. Random samplings of shoot apices collected at these periods from clones corresponding to these materials [3] were fixed then prepared according to standard histological procedures required for microscopic observations [4]. 30 median longitudinal apex sections, 7 μm thick, stained with Azure B [4], were examinated for each of the 4 age × vegetative state combinations. Every apical dome outline observed through the microscope was reported in an orthonormal (Ox, Oy) axis system — y-axis and the origin O corresponding respectively to the central longitudinal axis of the shoot apex and to its summit — to be experimentally defined by (x_i, y_i) coordinates.

As suggested by Schüepp [5], computer data processing established that the profiles observed could be reliably $-0.96<R^2<1$, [6]—described by a curvilinear regression of the form: $y=ax^b$.

A two-way analysis of variance [6] pointed out significant influences — $p<0.001$ — of the age and of the vegetative state of the plant material on the coefficient a, the exponent b and the product $a.b$, which defined each profile; moreover, a noticeable interaction — $p<0.001$ — of the two experimental factors was noticed for a and $a.b$. Statistical analysis results are detailed in Table.

The profiles characterizing each of the 4 apex samples are drawn in Figure 1, and correspond to the following regressions:

— juvenile clone, rest phase (*Fig.* 1 *a*): $y=0.41\ x^{1.60}$ ($R^2=1$);

— juvenile clone, budbreak (*Fig.* 1 *b*): $y=0.41\ x^{1.34}$ ($R^2=0.99$);

Note présentée par Alexis MOYSE.

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— mature clone, rest phase (*Fig.* 1 *c*): $y = 0.57\ x^{1.80}$ ($R^2 = 1$);

— mature clone, budbreak (*Fig.* 1 *d*): $y = 0.46\ x^{1.51}$ ($R^2 = 0.99$).

The analytic model adopted appeared to be the most satisfactory among the other regression forms tested to express, through a concise mathematic formulation, the conformation of vegetative shoot apices of giant sequoias.

The realistic profiles thus obtained showed that meristems from young trees were characrerized by a large basal surface, by contrast with those from mature trees, which appeared to be sharper and narrower, as illustrated in Figure 2. These observations are in accordance with similar studies referring to other coniferous species ([7], [8], [9]) and considering the ratio of the height on the diameter measurements of the apical dome, with the smaller values corresponding to the more juvenile forms.

The other main feature of our study was the influence of the vegetative state of the bud on the relevant apex morphology ([10], [11], [12]). In agreement with Owston on *Pinus strobus* [8], it was found that meristems of giant sequoia at the budbreak time enlarged by comparison with the rest phase conformation, and thus tended to have the profiles associated with the juvenile forms which provided the best result in *in vitro* cultures [2]. This phenomenon, which was particularly noticeable for mature trees, suggest to consider the evolutionary aspects of apical meristems during the year, in relation to seasonal variations. Moreover, keeping in mind the relationship between the morphological aspects and the organogenic capacities [2], meristem conformation could reflect inner basic characteristics depending on the age of the plant and on its vegetative state. Relevant cytomorphological analysis taking into account these two factors are underway, and would give soon complementary information to verify this hypothesis.

Nevertheless, from now on, the results presently reported appear to be helpful to select the most promising explants for the success of *in vitro* meristem culture.

INTRODUCTION. — En dépit de nombreux succès dans le domaine horticole, la culture *in vitro* de méristèmes de certaines espèces arborescentes à des fins de clonage demeure aléatoire [1]. Sur *Sequoiadendron giganteum* Buchholz, nous avons pu constater que l'influence du végétal s'exerce principalement à travers son âge et l'état physiologique des explants au moment du prélèvement, les meilleurs résultats étant obtenus *in vitro* à partir de méristèmes excisés de bourgeons de jeunes individus en train de débourrer [2]. Ces remarques nous ont incités à tenter de caractériser de la façon la plus simple et immédiate possible les échantillons de méristèmes de séquoia géant observés en culture *in vitro* en fonction de leur origine. L'analyse de contours de dômes méristématiques d'individus jeunes et âgés durant les stades phénologiques bien distincts de repos végétatif et de débourrement a été entreprise dans cette optique.

MATÉRIEL ET MÉTHODES. — *Origine des échantillons.* — Le matériel jeune, issu du bouturage d'un semis de séquoia géant âgé de 18 mois, peut être propagé par culture de méristèmes beaucoup plus facilement que le matériel âgé, provenant d'un séquoia géant centenaire greffé [2]. Ces pieds-mères, obtenus 3 ans auparavant [3] et cultivés depuis lors à l'extérieur, à l'Université de Clermont-Ferrand, présentent un port buissonnant diffus favorisé par des prélèvements fréquents. Ces plants sont représentatifs des deux classes d'âges considérées ([2], [3]). Les méristèmes primaires caulinaires observés proviennent d'extrémités d'axes végétatifs récoltés de façon aléatoire durant le repos végétatif de janvier, puis début mai, lors du débourrement.

Préparation des échantillons. — Les extrémités caulinaires débarrassées de la quasi-totalité de leurs formations foliaires sont fixées dans le mélange éthanol 95 %/formaldéhyde/acide acétique (17/2/1; v/v/v), puis déshydratées par une série d'alcool-toluène [4] avant inclusion dans la paraffine. Les coupes longitudinales de 7 µm d'épaisseur observées en microscopie optique ont été colorées par l'Azur B [4], en prévision d'analyses complémentaires ultérieures.

TABLEAU

Analyses de variance appliquées au cœfficient a, à l'exposant b et au produit $a.b$ (Plan d'ordre 2 à 30 répétitions).

Analysis of variance related to the cœfficient a, the exponent b and the product a.b (2^2 factorial design with 30 replicates).

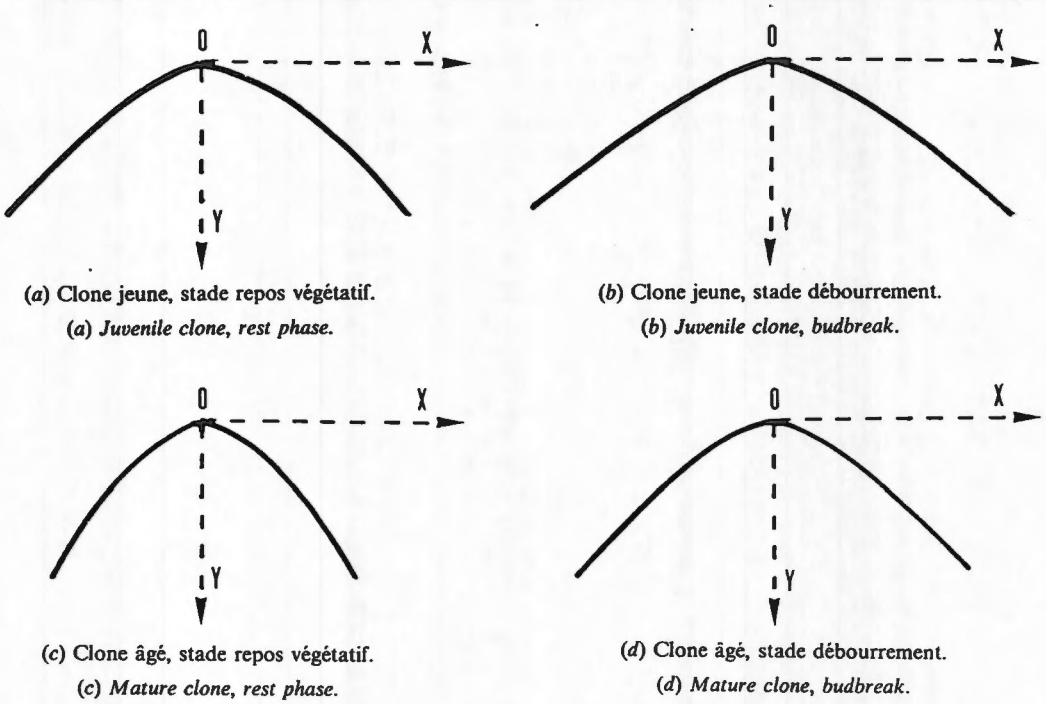
Origine	Paramètre considéré	Somme des carrés	Degré de liberté (d.d.l.)	Variance	Rapport de variances F
Entre stades végétatifs (repos ou débourrement)	a	0,104 2	1	0,104 2	12,40
	b	1,623 1	1	1,623 1	29,24
	$a.b$	1,324 4	1	1,324 4	69,71
Entre clones (jeune ou âgé)	a	0,278 0	1	0,278 0	33,10
	b	1,202 3	1	1,202 3	21,66
	$a.b$	1,914 7	1	1,914 7	100,77
Interaction	a	0,111 9	1	0,111 9	13,32
	b	0,014 3	1	0,014 3	0,25
	$a.b$	0,440 3	1	0,440 3	23,17
Résiduelle (directe)	a	0,976 4	116	0,008 4	–
	b	6,437 7	116	0,055 5	–
	$a.b$	2,209 4	116	0,019 0	–
TOTAL	a	1,470 5	119	–	–
	b	9,277 4	119	–	–
	$a.b$	5,888 8	119	–	–

Méthodes d'analyse, mesures et traitement des données. — La section la plus médiane de chaque méristème, débité longitudinalement en ruban de coupes, est choisie pour reporter au moyen d'une chambre claire le contour du dôme méristématique sur une feuille de papier, en précisant l'échelle. 30 tracés pour chaque combinaison : âge du clone (jeune ou âgé) × stade végétatif (repos ou débourrement), soit au total 120 figures, ont été analysés.

Des observations réalisées tant dans l'espace qu'à partir de coupes longitudinales et transversales, tendent à assimiler la configuration des dômes méristématiques des points végétatifs caulinaires de séquoia géant, comme pour d'autres végétaux [5], à des paraboloïdes elliptiques de révolution. Le contour de chacun des méristèmes observés peut ainsi être caractérisé en projection plane à partir d'un ensemble de coordonnées cartésiennes (x_i, y_i) déterminées graphiquement dans un quadrant d'un repère orthonormé (Ox, Oy), l'origine O et l'axe y des ordonnées correspondant respectivement au sommet et à l'axe central longitudinal du méristème considéré, comme il est indiqué dans la figure 1. Le traitement informatique des données obtenues semble indiquer que chaque relevé peut être décrit par une régression curvillinéaire de type puissance, de la forme $y=ax^b$, le coefficient de détermination R^2 renseignant sur la qualité de l'ajustement établi. Sur ces bases, les effets de l'âge du clone et du stade végétatif ont été analysés à travers le cœfficient a, l'exposant b, et le produit $a.b$, au moyen d'analyses de variance à deux critères de classification appliquées à l'expérience factorielle 2^2 (plan d'ordre 2 à 30 répétitions) d'ensemble [6]. Une illustration synthétique de ces résultats sous forme d'un profil type de chacun des quatre échantillons de méristèmes considérés est proposée à partir des valeurs moyennes des couples (x_i, y_i) correspondant au 30 contours; le tracé intégral est obtenu par symétrie par rapport à l'axe Oy de l'ajustement établi pour le demi-axe Ox.

RÉSULTATS. — L'examen de l'ensemble des régressions effectuées pour les 120 cas de figures confirme que x et y sont liés de façon très satisfaisante par une relation de la forme $y=ax^b$, R^2 variant de 0,96 à 1.

Les différentes analyses de variance résumées dans le tableau révèlent un effet hautement significatif ($p<0,001$) des facteurs : âge du clone et stade végétatif, sur les valeurs de a, de b, et de $a.b$, caractérisant chaque contour méristématique. L'influence de l'interaction entre les deux facteurs expérimentaux considérés se ressent très nettement sur le cœfficient a et le produit $a.b$ ($p<0,001$).



(*a*) Clone jeune, stade repos végétatif.
(*a*) *Juvenile clone, rest phase.*

(*b*) Clone jeune, stade débourrement.
(*b*) *Juvenile clone, budbreak.*

(*c*) Clone âgé, stade repos végétatif.
(*c*) *Mature clone, rest phase.*

(*d*) Clone âgé, stade débourrement.
(*d*) *Mature clone, budbreak.*

Fig. 1. — Profils caractéristiques de chacun des quatre échantillons de méristèmes étudiés (G × 570)
Fig. 1. — Characteristic outlines of the apical domes corresponding to the four different origins observed (M × 570).

Les profils caractéristiques de chacun des quatre échantillons de méristèmes étudiés sont regroupés au sein de la figure 1. Les tracés présentés ont été réalisés à partir des régressions suivantes ($x \geqq 0$) :

clone jeune, stade repos végétatif (*fig.* 1 *a*) : $y = 0{,}41\ x^{1{,}60}$ ($R^2 = 1$);
clone jeune, stade débourrement (*fig.* 1 *b*) : $y = 0{,}41\ x^{1{,}34}$ ($R^2 = 0{,}99$);
clone âgé, stade repos végétatif (*fig.* 1 *c*) : $y = 0{,}57\ x^{1{,}80}$ ($R^2 = 1$);
clone âgé, stade débourrement (*fig.* 1 *d*) : $y = 0{,}46\ x^{1{,}51}$ ($R^2 = 0{,}99$).

Discussion. — Le choix de la fonction $y = ax^b$ pour caractériser les profils mérisrématiques de séquoia géant étudiés s'explique par l'intensité de la liaison tout à fait satisfaisante entre les variables x et y, comme l'illustrent les valeurs de R^2 obtenues; en outre, cette relation séduit par sa formulation concise par rapport à d'autres types de régression éprouvés qui n'ont pas permis d'améliorer la qualité de l'ajustement. La méthodologie employée, à travers le modèle analytique retenu, traduit de façon claire et réaliste la conformation des extrémités apicales des points végétatifs, en relation avec la morphogenèse, sans sous-estimer la variabilité intra-échantillon illustrée par la variance résiduelle.

Comme pour *Picea abies* [7], les dômes méristématiques évasés, reposant sur une grande surface basale, semblent refléter un certain état juvénile (*fig.* 2), qui s'exprime notamment par une bonne aptitude à la multiplication végétative, en rapport avec une morphologie foliaire bien développée [3]. En revanche, les contours méristématiques plus pointus correspondent aux formes âgées, caractérisées entre autres, par des feuilles très réduites et une inaptitude au clonage ([2], [3]). Ces résultats concordent avec les observations réalisées sur *Pinus strobus* [8] et *Pinus ponderosa* [9], en considérant le rapport des

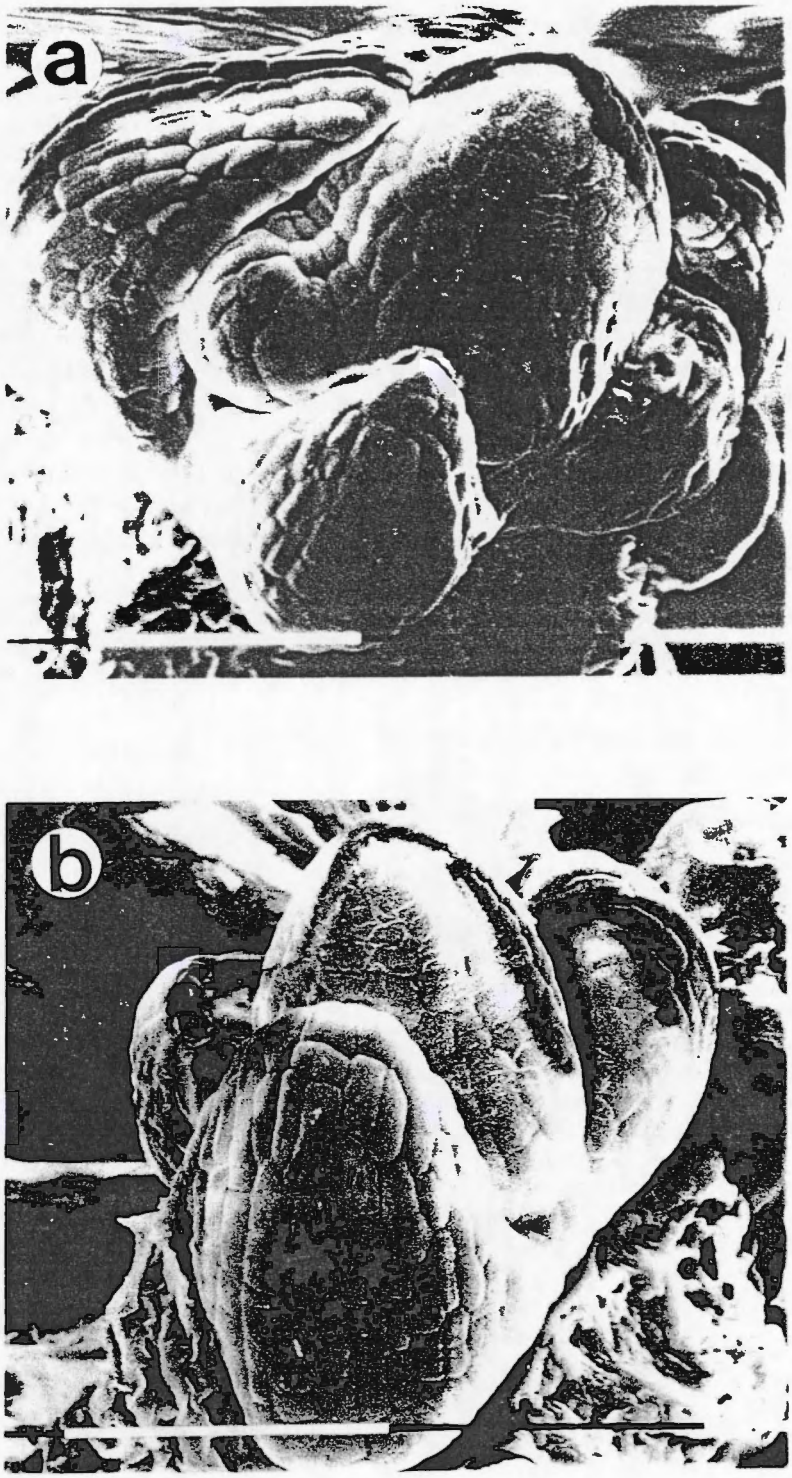


Fig. 2. — Méristèmes apicaux caulinaires de *Sequoiadendron giganteum* observés durant le repos végétatif. Le dôme méristématique du clone juvénile (*a*) est plus évasé que pour le clone âgé (*b*); échelle : 100 µm.

Fig. 2. — Shoot apical meristems of Sequoiadendron giganteum *observed during rest phase. The apical dome of the juvenile clone* (*a*) *looks wider than the mature clone one* (*b*)*; scale bar:* 100 µm.

mesures de la hauteur et du diamètre des méristèmes prélevés sur des individus jeunes et âgés.

Par ailleurs, l'évolution des profils méristématiques en fonction de la phénologie des bourgeons ([10], [11], [12]), présentement mise en évidence pour des stades végétatifs bien distincts, incite à considérer l'aspect évolutif de certaines caractéristiques méristématiques, dont la forme ne pourrait être qu'un indice [10], et qui seraient influencées par des paramètres saisonniers. Conformément aux observations de Owston sur *Pinus strobus* [8], les méristèmes de séquoia géant ont tendance à sévaser au stade débourrement, évoluant par là même vers le profil méristématique caractéristique des formes juvéniles. Ce phénomène est particulièrement important pour les méristèmes de séquoias géants âgés, dont les différences avec leurs homologues issus de jeunes sujets ont tendance à s'atténuer au moment du débourrement, notamment en ce qui concerne leur réactivité en culture *in vitro* très nettement supérieure à l'ordinaire [2].

Des investigations en cours devraient fournir très prochainement des informations complémentaires, sur le plan cytologique, aux observations morphologiques exposées qui permettent, dès à présent, de choisir lors de la mise en culture les méristèmes les plus prometteurs en vue du clonage.

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In vitro meristem culture of juvenile and mature *Sequoiadendron giganteum*

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Summary

A total of 7000 meristems were used in experiments to investigate the possibility of cloning *Sequoiadendron giganteum* Buchholz by *in vitro* meristem culture of juvenile (2-year-old) and mature (100-year-old) ortets. Cultures were initiated on a low-salt medium containing 0.1 mg l^{-1} 1-naphthaleneacetic acid to stimulate meristematic activity. Benzylamino purine (0.01–0.5 mg l^{-1}) inhibited meristematic activity, whereas gibberellic acid (0.01–0.5 mg l^{-1}) had no effect on meristematic development. The mature ortet showed more specific mineral requirements and a lower capacity for cloning than the juvenile ortet. Rooted plants were obtained only from the juvenile clone. There was a marked seasonal effect on meristematic activity, especially for the mature clone, the most active material being obtained during budbreak.

Introduction

There is much interest in the use of cloning in forestry (Zobel 1981). The success of clonal reforestation programs is limited by the efficiency with which selected trees can be reproduced vegetatively (Durzan 1984, Bonga 1987). Woody species in the juvenile phase are generally easy to clone by conventional techniques. The ease with which many trees are propagated tends to diminish, however, as they approach a size that is sufficient to allow a reliable evaluation of their crop potential (Mott 1981, Bonga 1982*b*). *Sequoiadendron giganteum* Buchholz, the giant sequoia, is no exception to this rule (Monteuuis 1985). The shoot apical meristems are presumed to play a key role in phase change phenomena (Bonga 1982*b*) and consequently in the cloning potential of mature ortets (Margara 1982, Nozeran 1983, Bonga 1987, Franclet et al. 1987). To study phase change phenomena and cloning potential in more detail we have examined the meristematic behavior of explants of juvenile and mature giant sequoias. Here we describe the culture conditions leading to plantlet regeneration from excised meristems of giant sequoia, and compare the cloning potential of juvenile and mature material.

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Materials and methods

Plant material

The mature clone was obtained by grafting scions from a 100-year-old ortet onto young giant sequoia seedlings. The juvenile clone consisted of rooted cuttings derived from a two-year-old seedling. Details of the procedures are reported in a previous paper (Monteuuis 1985) which established that the juvenile clone (clone 45 115) was easy to propagate by cuttings whereas the grafted mature clone (clone 15) could not be propagated by conventional techniques. The cloned material was grown for two years either outdoors at the University of Clermont-Ferrand (France) or in controlled environment chambers (Monteuuis and Bon 1986).

Stem segments from the cloned stock plants were surface sterilized by dipping briefly in 70% ethyl alcohol before the shoot apical meristems were excised aseptically under a cold light source and immediately placed on initiation medium. In this paper, the term meristem includes the apical dome and the youngest leaf primordia; any surrounding young leaves were removed. Such explants did not exceed 200–250 μm in diameter.

Unless otherwise stated, experiments were with explants from the mature clone growing in a controlled environment chamber.

In vitro culture conditions

Each culture medium was adjusted to pH 5.5 with 0.1 M KOH before the addition of agar and then autoclaved at 120 °C, except for the initiation medium which was sterilized at 110 °C. Four ml of medium was aseptically poured into each 35-mm wide plastic tissue culture dish and five excised meristems were then placed in each dish. The dishes were sealed with Parafilm® and maintained in a growth chamber at 20 °C with 'Mazda Fluor Lumière du jour' fluorescent lamps providing 14 W m^{-2} irradiance at culture level during the 16-h photoperiod. After the three weeks required for initiation on the LP/2 basal medium (see Table 1 for composition), cultures were transferred monthly to LP/4 medium (see Table 1) until they were about 1 mm tall, this stage took from one to nine months depending on the vigor of the explant. Explants were then transferred to elongation medium (EM, see Table 1). When the shoots were 10 to 15 mm tall they were placed on a low salt and sucrose medium containing 1 mg l^{-1} NAA for three weeks. To initiate root development, the shoots were then transferred to the same medium lacking growth regulators (Monteuuis and Bon 1986). On this medium 80 to 100% of the explants from the juvenile clone formed roots. The rooted microcuttings were acclimated outdoors.

Experimental design

A total of 7000 excised meristems were tested under various *in vitro* culture conditions. Only the treatments having a major effect on the establishment of the meristem cultures are reported here. Experiments involved at least two samplings per treatment, each sampling comprised three replications of 15 meristems.

Macronutrient experiments

Meristem cultures were initiated on standard media such as those of Murashige and Skoog, Gamborg and Lepoivre (Quoirin et al. 1977, Augé et al. 1982. Margara 1982). In addition, four low-salt macromineral compositions (see Table 1) were tested. Seventy-five explants (5 samplings each of 15 meristems) were used for each macronutrient × clone combination.

Growth regulatory experiments

The growth regulators, benzylamino purine (BAP) and gibberellic acid (GA_3) were tested at 0.01, 0.1 and 0.5 mg l^{-1} alone or mixed with 0.01, 0.05 or 0.1 mg l^{-1} of 1-naphthaleneacetic acid (NAA) in the LP/2 basal initiation medium.

Meristem response measurements and statistical methods

After three weeks' culture on initiation medium, meristem reactivity (MR) was estimated by expressing the number of chlorophyllous organogenic explants observed as a percentage of the total number of explants. Results were analyzed by the chi-squared test (χ^2) or the analysis of variance test (F-test) after Arcsin $\sqrt{x}$ angular transformation (Snedecor and Cochran 1957). Treatments were considered significant for P values ≤ 0.05. The data are expressed ± confidence interval for $P = 0.05$.

Results and discussion

Macronutrients

In contrast to the meristems of angiosperms (Smith and Murashige 1970, Pennazio and Vecchiati 1976, Scaramuzzi and D'Elia 1984), those of *Sequoiadendron giganteum* cultured on high salt media (macronutrient concentrations of 40–50 mM) such as Murashige and Skoog, Gamborg or Lepoivre full-strength solutions did not undergo organogenesis. On these media, meristem cultures showed necrosis, vitrification and other degenerative symptoms of high salinity (Beauchesne 1981, Margara 1982). Organogenesis by *Sequoiadendron giganteum* meristem cultures was induced, however, on relatively simple media (GB/2, LP/2, HR and KP in Table 1) lacking growth substances and with a total salt concentration of 20–30 mM (Figure 1). Similar observations have been reported for meristem cultures of other gymnosperms including *Picea abies* (L.) Karst. (Romberger and Tabor 1971), *Pseudotsuga menziesii* (Mirb.) Franco (Bekkaoui et al. 1985, Bekkaoui 1986), *Sequoia sempervirens* (D. Don) Endl. and *Pinus pinaster* Ait. (Walker et al. 1985).

Exogenous growth regulators

Tests with explants of the mature clone showed that organogenesis was strongly promoted by NAA. At concentrations of 0.1, 0.01 and 0 mg l^{-1}, meristem reactivity (MR) scores were 74 ± 7%, 55 ± 8% and 28 ± 7%. In a comparison of the

Table 1. Compositions of the culture media used for cloning giant sequoias by *in vitro* meristem culture (mg l^{-1}).

	GB/2[1]	HR[1]	KP[1]	LP/2[1]	LP/4	EM[2]
Macronutrients						
NH_4NO_3	–	–	–	200	100	825
KNO_3	1250	–	250	900	450	950
$CaCl_2.2H_2O$	75	75	–	–	–	220
$MgSO_4.7H_2O$	125	250	250	180	90	185
KH_2PO_4	–	–	250	135	67	85
KCl	–	750	–	–	–	–
$NaH_2PO_4.H_2O$	75	125	–	–	–	–
$Ca(NO_3)_2.4H_2O$	–	–	1000	600	300	–
$(NH_4)_2SO_4$	67	–	–	–	–	–
$NaNO_3$	–	600	–	–	–	–
Micronutrients						
$FeSO_4.7H_2O$	27.8	27.8	27.8	27.8	13.9	27.8
Na_2-EDTA	37.2	37.2	37.2	37.2	18.6	37.2
$CoCl_2.6H_2O$	0.025	0.025	0.025	0.025	0.025	0.025
$CuSO_4.5H_2O$	0.025	0.025	0.025	0.025	0.025	0.025
$MnSO_4.4H_2O$	22.3	22.3	22.3	22.3	22.3	22.3
KI	0.83	0.83	0.83	0.83	0.83	0.83
$Na_2MoO_4.2H_2O$	0.25	0.25	0.25	0.25	0.25	0.25
$ZnSO_4.7H_2O$	8.6	8.6	8.6	8.6	8.6	8.6
H_3BO_3	6.2	6.2	6.2	6.2	6.2	6.2
Organic constituents						
Myo-inositol	100	100	100	100	100	50
Thiamine-HCl	1	1	1	1	1	1
Pyridoxine-HCl	1	1	1	1	1	1
Glycine	2	2	2	2	2	2
Nicotinic acid	1	1	1	1	1	1
Sucrose	30000	30000	30000	30000	30000	30000
Bacto agar (Difco)	8000	8000	8000	8000	8000	7000
Activated charcoal (Merck 2186)						20000

[1] Gamborg, GB/2; Heller, HR; Knop, KP; and Lepoivre, LP/2 (Quoirin et al. 1977, Augé et al. 1982). We used LP/2 as the basal medium.
[2] Elongation medium, EM.

effects of NAA, IBA and IAA at a concentration of 0.1 mg l^{-1}, inititiation percentages were 66 ± 11%, 50 ± 11% and 23 ± 10%, respectively. All concentrations of BAP that were tested reduced organogenesis by meristems cultured in LP/2 from a control value of about 25% to between 5 and 10%. In the presence of 0.1 mg l^{-1} NAA, 0.5 mg l^{-1} BAP reduced organogenesis from 84 ± 13% to 0%. At all concentrations tested, GA_3 had no effect on organogenesis in either the presence or absence of NAA. The promotion of organogesis by NAA in giant sequoia meristems is consisent with the results of similar studies with *Sequoia sempervirens* (Verschoore-Martouzet 1985) and potato (*Solanum tuberosum* L.) (Pennazio and Vecchiati 1976). According to Smith and Murashige (1970) isolated meristems from angiosperms contain insufficient endogenous auxin to support

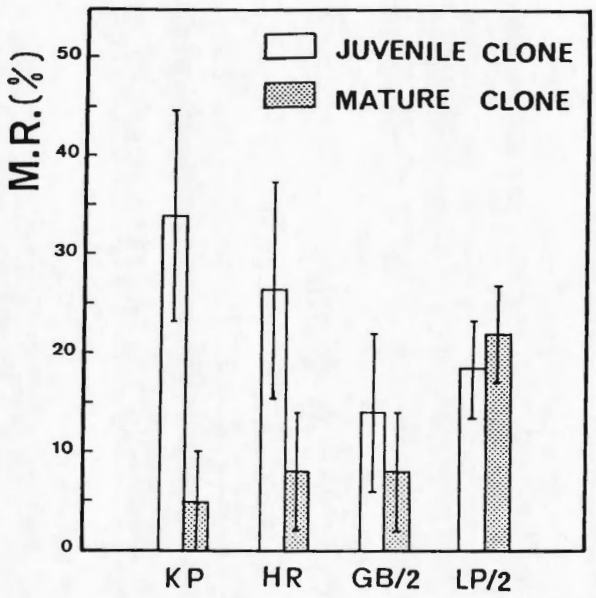

Figure 1. Comparative effects of four different macronutrient formulations (see Table 1) on meristem reactivity (MR) during initiation of *in vitro* meristem cultures. Vertical bars represent confidence intervals at $P = 0.05$ level.

meristem initiation. However, explants of *S. giganteum* initiated on NAA needed transferring to an auxin-free medium (LP/4) to avoid toxicity symptoms as found for other species of gymnosperm (Mott 1981, Margara 1982).

Tissue maturity

Percent meristem initiation varied according to the macromineral formulation, which had the greatest influence on mature material ($0.001 < P < 0.01$) (Figure 1). This indicates that the macromineral requirements of the cultures become more specific with explant maturity as has been reported to be the case in other species (Berlyn and Beck 1980, Margara 1982, Yilmaz-Lentz 1984). Best results with mature meristems were obtained on LP/2 medium. This formulation was adopted initially to improve meristem cultures of *Prunus* spp. by reducing browning and necrosis (Quoirin et al. 1977), and was later found useful with meristem cultures of other species (Bekkaoui 1986, Texier and Faucher 1986). The superior results obtained with this medium may be related to its low ammonium content (cf. Margara 1978 and Bekkaoui 1986).

On the LP/2 medium, meristem reactivity of the mature clone was higher than that of the juvenile clone. In absolute terms, enhancement of initiation by 0.1 mg l^{-1} NAA was greater in the mature, than the juvenile, clone ($0.001 < P < 0.01$) (Figure 2). A greater stimulation of initiation by auxin in mature than juvenile meristems has also been reported in *Sequoia sempervirens* (Verschoore-Martouzet 1985). Rooting of shoots obtained from the juvenile clone occurred on the elonga-

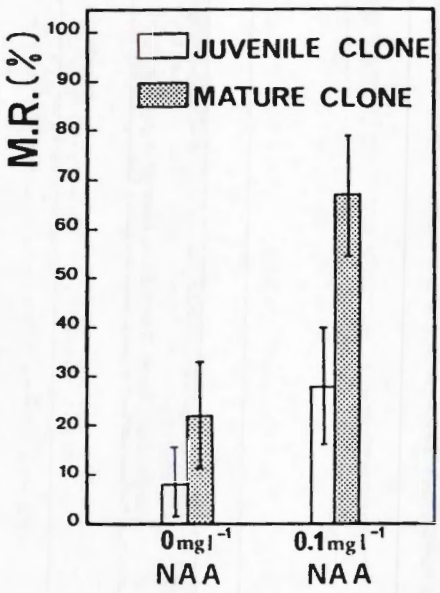

Figure 2. Effects of NAA on meristem reactivity (MR) of the juvenile and mature clones during initiation of cultures on LP/2 medium. Vertical bars represent confidence intervals ($P = 0.05$).

tion medium (EM), although many more shoots rooted (80–100%) on the rooting medium described by Monteuuis and Bon (1986). Only a few shoots of the mature clone reached a stage at which they could be tested for rooting.

Seasonal variation

In the absence of NAA, percent organogenesis on LP/2 was almost twice as high (51 *versus* 27%) in meristems excised in May, at the time of budbreak, than in meristems excised in January during the dormant period (Figure 3). Organogenesis was correspondingly less dependent on exogenous auxin in meristems from breaking buds than in meristems excised during the rest period (Figure 3). As observed in other species (Jonard et al. 1983, Meynier 1985), the physiological stage of the donor plant also affected the later development of the cultures. Following transfer to LP/4 medium, initiated meristems taken from breaking buds had a lower failure rate than meristems cultured from resting buds (60–65% *versus* 90–95% for the mature clone). Transplanted cultures originating from breaking buds also had a shorter quiescent phase—a few weeks—preceding the onset of active growth than those from resting buds—up to 8–9 months). Morphogenetic differences between cultured shoots from juvenile and mature meristems were least when the cultures were established with meristems from breaking buds (see Bonga 1987). Currently the possibility of propagating mature clones by rooting cuttings from shoots rejuvenated *in vitro* from meristems excised during budbreak is under investigation.

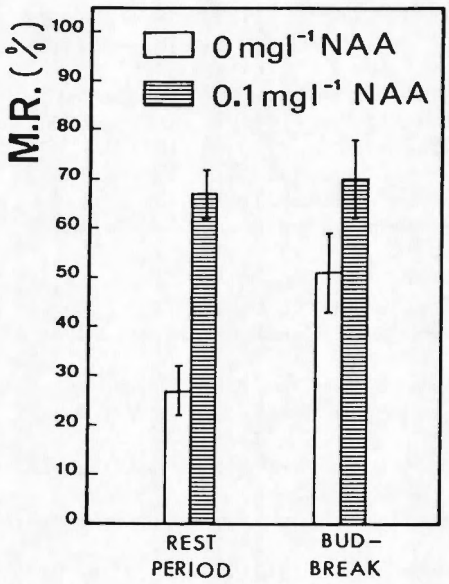

Figure 3. Effects of exogenous NAA and developmental stage of the buds from which meristems were excised on *in vitro* meristem reactivity (MR) on LP/2 initiation medium. Vertical bars represent confidence intervals ($P = 0.05$).

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Nucleotide and nucleic acid status in shoot tips from juvenile and mature clones of *Sequoiadendron giganteum* during rest and growth phases

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Summary

Nucleoside triphosphate and nucleic acid contents of shoot tips of juvenile and mature clones of *Sequoiadendron giganteum* Buchholz were analyzed during rest and growth phases. In both juvenile and mature clones, shoot growth activity was characterized by significant increases in ATP, non-adenylic nucleoside triphosphate (NTP) and RNA levels. During the rest period, both ATP/NTP and RNA/DNA ratios were significantly higher in the juvenile clone than in the mature clone. However, during the growth phase, only the ATP/NTP ratio was higher in the juvenile than in the mature clone. The results suggest that the physiological differences between shoot tips of juvenile and mature tissues during the rest phase tend to decline as active shoot growth commences. This conclusion is consistent with morphological observations and with the varying organogenetic capacities of *in vitro* cultures of explants removed from stock plants at different times.

Introduction

Aging in woody plants is closely associated with a decreased capacity for vegetative regeneration. This presents problems in the cloning of selected forest trees because when the ortets reach a size large enough to demonstrate their potential, they have lost their regenerative capacities.

Organogenesis of explants of *Sequoiadendron giganteum* Buchholz is mainly dependent on the age of the ortet and on its physiological state at the time of collection. However, the mechanisms underlying the differences in organogenic capacities observed between juvenile and mature clones in relation to shoot growth activity are unknown. The studies of Gendraud and Prévôt (1973), Gendraud (1975), Riding (1976) and Davies (1984) on changes in nucleotide and nucleic acid metabolism appeared to be promising and were therefore extended to our experimental material.

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Materials and methods

Plant material

The juvenile clone of *Sequoiadendron giganteum* consisted of cuttings derived from a two-year-old seedling. The difficult-to-root, 100-year-old clone was obtained by grafting scions collected from a 100-year-old ortet onto young seedling rootstocks. These two categories of stock plants were obtained two years ago and were cultivated outdoors under identical conditions at the University of Clermont-Ferrand, France.

Sample collection

Samples were collected on five dates in both January and May 1986 and corresponded with the rest phase and the onset of the growth phase, respectively, of the stock plants. As each sample was removed, the temperature was noted; the means were 5 °C (from −1 °C to 14 °C) for January collections and 15 °C (from 7 °C to 20 °C) for May collections. Biochemical analyses were carried out on samples of 15 to 20 shoot tips that included the apical meristem and some young non-chlorophyllous leaves and a small piece of underlying tissue. The shoot tips did not exceed 1 to 2 mm in length. Shoot tips were excised under a cold light source and immediately placed on moistened Whatman paper at 0 °C in darkness until the whole sample had been processed (15 min), and then weighed (about 25 mg fresh weight).

Nucleotide determination

Juvenile and mature clone samples were collected and analyzed under identical conditions. Immediately after a sample had been weighed it was homogenized with 5 volumes of frozen 0.6 N perchloric acid and then centrifuged (0 °C). The pellet was saved and assayed for RNA and DNA. The supernatant was adjusted to pH 8.5 with solid $KHCO_3$. The $KClO_4$ was removed by centrifugation at 0 °C (Keppler et al. 1970). Aliquots of the neutralized supernatant were used to assay for ATP and non-adenylic nucleoside triphosphate (NTP) by the luciferin luciferase (Boehringer Mannheim) technique with a Jobin Yvon bioluminometer in accordance with Pradet (1967) and Gendraud (1977). The nucleoside diphosphate kinase was obtained from Boehringer Mannheim. All manipulations were performed quickly under standard conditions (cold, darkness) to prevent phosphate-linkage hydrolysis.

DNA and RNA determination

The sample pellet was rinsed with 0.6 M perchloric acid followed by 95% ethanol and acetone and stored at −25 °C until analyzed for nucleic acids. After KOH hydrolysis and neutralization, RNA was assayed spectrophotometrically at 260 nm (Pye Unicam SP8-100), according to Schmidt and Tannhauser (1945). Deoxyribonucleic acid was estimated by the Dische (1930) colorimetric method.

Statistical analysis

The influence of the season in relation to the shoot vegetative state (rest or growth phase) was determined by comparing all data obtained during the winter with those obtained in the spring by means of the analysis of variance test (F-test, Snedecor and Cochran 1957). The Wilcoxon test (Snedecor and Cochran 1957) was used to compare juvenile and mature clone measurements. Data variability is expressed by vertical bars representing a confidence interval at $P = 0.05$. In all statistical analyses, differences were considered significant for P values ≤ 0.05.

Results

Nucleoside triphosphates

By comparison with the rest phase, shoot growth activity in both clones was characterized by a significant increase in the nucleoside triphosphate pool as represented by ATP ($P < 0.001$) and NTP ($P < 0.001$) (Figure 1).

Because of the wide variation within clones, the ATP/NTP ratios of the juvenile and mature clones were paired by date before analysis (Table 1). The ATP/NTP values were significantly ($P < 0.001$) greater in the juvenile clone than in the mature clone for tissue collected during the rest period, but this response was less marked ($0.01 < P < 0.025$) in tissues collected during the growth period. Means for the rest and growth periods indicate that ATP/NTP tended to decrease in the juvenile clone as the shoot began to grow, whereas it increased in the mature clone. Ratios of ATP/(ATP + NTP) provided a more reliable measure of inter-clonal differences in relation to the vegetative stage of the shoot. During the rest period, in spite of greater variation in absolute values, ratios were generally higher

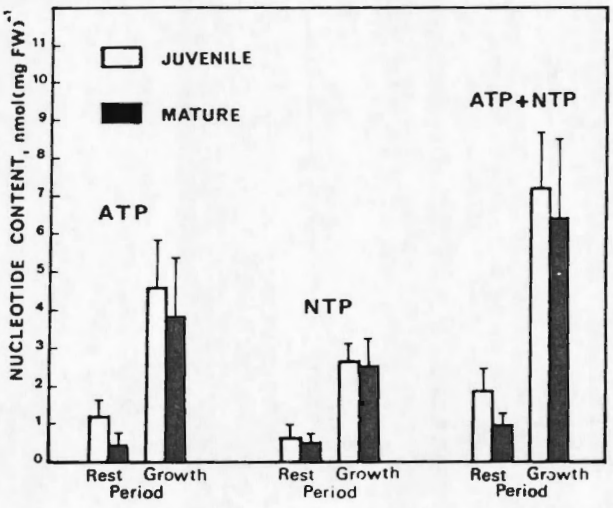

Figure 1. Shoot phenology and the mean nucleotide content of juvenile and mature clones. Vertical bars represent confidence intervals ($P = 0.05$).

Table 1. Comparisons of ATP/NTP ratios of juvenile and mature clones in relation to shoot phenology. Results are expressed as pairs of values (x, y) with reference to the time of sample collection; x denotes the juvenile clone value and y the mature clone value. The Wilcoxon test established that the differences between the two clones was more marked ($P < 0.001$) during the rest period than during the growth period ($0.01 < P < 0.025$).

Collection period	ATP/NTP pairs of values for juvenile and mature clones	Means ($\bar{x} \pm SE, \bar{y} \pm SE$)
Rest period	(2.5, 1.8) (1.2, 0.9) (3.2, 0.8) (4.0, 1.6) (2.6, 0.4) (2.0, 1.3) (0.7, 0.3) (0.8, 0.5)	2.1 ± 1.2, 0.9 ± 0.6
Growth period	(2.9, 2.2) (1.6, 1.5) (1.6, 1.3) (2.5, 1.9) (1.1, 1.0) (1.9, 2.1) (1.5, 1.3) (1.5, 1.2) (1.2, 1.4) (1.7, 1.3)	1.8 ± 0.6, 1.5 ± 0.4

for the juvenile clone than for the mature clone (Figure 2). In contrast, there were no significant differences in ATP/(ATP + NTP) values between the juvenile and the mature material during the growth phase.

Nucleic acids

In both clones, RNA content was significantly ($P < 0.001$) higher during the

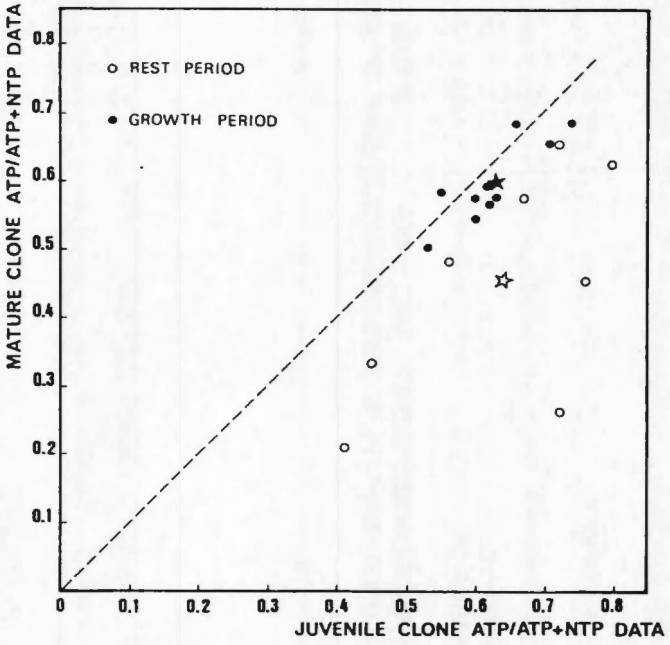

Figure 2. Ratios of ATP/(ATP + NTP) for juvenile and mature clones. The points represent the results obtained on different dates during the rest (January) and growth (May) periods. The stars represent the means for all dates: rest period (☆); and growth period (★). The broken line indicates where the points would lie if the ratios were the same in the juvenile and mature clones.

period of shoot growth than during the rest period (Figure 3), whereas DNA concentrations did not alter with level of growth activity. When expressed as an RNA/DNA ratio, a significant interclonal difference ($P < 0.001$) was found only during the rest period when RNA/DNA ratios were higher in the juvenile clone (Table 2).

Discussion

Biochemical analysis of lignified material is difficult. This is particularly true for compounds such as nucleotides and especially for ATP and NTP which can be hydrolyzed quickly. Despite rigorous standardization of procedures, a large range in data was observed within clones, especially when samples were collected during the rest period. McKee and Mendelssohn (1984) reported similar fluctuations in adenylic nucleotide levels and suggested that changing environmental

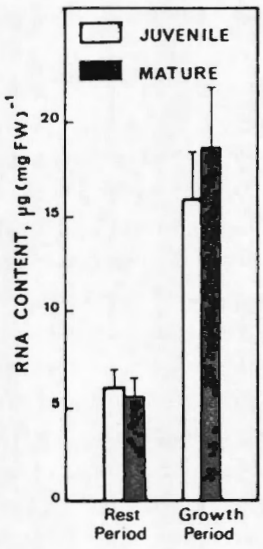

Figure 3. Shoot phenology and mean RNA content of juvenile and mature clones. Vertical bars represent confidence intervals ($P = 0.05$).

Table 2. RNA/DNA means ± SE of the juvenile and the mature clones with reference to shoot phenology at collection time. The difference between the two clones is highly significant ($P < 0.001$) for the rest period.

RNA/DNA (mean ± SE)	Rest period		Growth period	
	Juvenile clone	Mature clone	Juvenile clone	Mature clone
	0.394 ± 0.032	0.280 ± 0.028	1.650 ± 0.848	1.550 ± 0.644

conditions may be the cause. In the present study, natural variation in temperature and photoperiod could explain the variation in results obtained from one sampling date to another. For this reason values for juvenile and mature clones were paired for comparison according to the time of material collection.

The onset of shoot growth was associated with an increase in nucleoside triphosphate and RNA levels that may reflect a general metabolic stimulation attributable to the beginning of more favorable environmental conditions for growth (Gendraud 1975, Lavarenne et al. 1982, Thomas et al. 1985). The nucleoside triphosphate data were similar to those of Sagisaka (1981) and McKee and Mendelssohn (1984) who observed higher ATP levels during the growth period. Because ATP participates in many biochemical pathways, its concentration may reflect cellular metabolic potentialities. During the rest period in which temperatures averaged 5 °C, the juvenile clone was characterized by a higher ATP concentration than the mature clone. Perhaps, a higher ATP content facilitates metabolism at low temperatures. The ATP/NTP ratio declined in the juvenile clone as shoots began to grow because of an increase of the NTP level, whereas the ATP/NTP ratio increased in the mature clone, mainly because of a higher relative amount of ATP (75% more).

Shoot elongation was associated with a significant increase in RNA level, whereas DNA content was unaffected. This could be the result of enhanced RNA biosynthesis as reported by Gendraud and Prévôt (1973) for sprouting Jerusalem artichoke tubers and Davies (1984) for *Ficus pumila* L. The RNA/DNA values indicate that the time of collection in relation to shoot phenology (rest or growth period) influenced the results of juvenile and mature clone comparisons: the significant difference observed during the rest stage disappeared when shoots began to grow. Riding (1976) and Davies (1984) demonstrated that RNA levels were positively correlated to rooting potential, the juvenile, easy-to-root plants being characterized by high RNA values in contrast with the mature, difficult-to-root plants. Moreover, Davies (1984) found that during the growth period, RNA contents and rooting levels of the mature material increased relative to those of the juvenile clone. This author suggested that differences between juvenile and mature plants decreased as shoots began to grow. Moreover, best cloning results from *in vitro* meristem cultures of juvenile and mature clones of *Sequoiadendron giganteum* have been recorded when explants were removed at the beginning of the growth period (Monteuuis 1987). When cultures were initiated at this time, differences in vegetative regeneration capacities between the juvenile and the mature material were slight.

These results support Franclet's (1983) concept of phase change which emphasizes that shoot tip maturation processes occur during each growth phase, increasing simultaneously with shoot elongation, the most juvenile state corresponding with the beginning of active growth. Morphological indices of development such as leaf shape are consistent with this interpretation.

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Physiologie végétale/*Plant Physiology*

Application de la technique micro 2D PAGE au microgreffage de Sequoiadendron giganteum Buchholz

Marie-Claude Bon et Olivier Monteuuis

Résumé — Une nouvelle technique de microanalyse par électrophorèse bidimensionnelle, appliquée à des points végétatifs de séquoias géants centenaires greffés *in vitro* sur de jeunes semis, a permis d'observer le profil protéique du méristème terminal de greffons de type morphologique juvénile et mature. Les formes matures se différencient des formes morphologiquement rajeunies par une densité protéique plus importante, notamment du point de vue des protéines acides. Une interprétation de ces résultats est proposée, en envisageant les perspectives offertes par ce type d'analyse pour une étude physiologique du greffage.

Micro 2D PAGE technique applied to micrografting of *Sequoiadendron giganteum* Buchholz

Abstract — *A new microanalytic technique, 2D PAGE, applied to meristems of one hundred-year-old giant sequoias grafted onto young seedlings rootstocks* in vitro, *enables us to observe protein patterns of shoot apical meristem corresponding to either mature or juvenile scion morphology. The mature types differ from the juvenile ones through more abundant protein population especially from the acid protein view point. An interpretation of these results is proposed looking forwards to the advantages of such a technique to analyse the physiological basics of grafting.*

Introduction. — Le microgreffage *in vitro* d'extrémités végétatives caulinaires présente beaucoup d'attraits, notamment pour l'amélioration phytosanitaire des agrumes ([1], [2]). Cette technique est également appliquée pour faciliter le clonage d'arbres âgés ([3], [4], [5]). Ainsi sur *Sequoiadendron giganteum* Buchholz, la miniaturisation du greffon (200 µm) favorise la réacquisition rapide des caractéristiques de type juvénile [6] comme la morphologie foliaire, plus ou moins marquées et variables dans le temps en fonction des greffes considérées [7].

L'étude de ces phénomènes nécessite des moyens d'investigation biochimique adaptés aux dimensions très réduites des structures végétatives manipulées, en l'occurence un méristème. La technique d'électrophorèse 2D PAGE miniaturisée ([8], [9]), récemment mise au point pour ce type d'analyse, a permis d'observer les modifications de la composition protéique associées au microgreffage des points végétatifs de séquoia géant âgé sur de jeunes semis de la même espèce.

Matériel et méthodes. — *Le matériel végétal.* — La technique de microgreffage a été décrite antérieurement [(6], [7]). Les points végétatifs greffés sur de jeunes semis *in vitro* proviennent de plusieurs têtes de clones centenaires, individus n° 03 et n° 15 notamment [10]. Le méristème terminal du greffon est prélevé sous loupe binoculaire et lumière froide, lorsque ce dernier a atteint une taille de 0,5 à 1 mm, permettant l'observation et la caractérisation de la nouvelle morphologie, en distinguant le type juvénile du type mature (*fig.* 1). Ce méristème, constitué du dôme méristématique et de un ou deux primordiums, est rapidement broyé sur lame de microscope. L'extrait obtenu est resuspendu dans le milieu de lyse de Granier et de Vienne [11].

2D PAGE. — L'isoélectrofocalisation (IEF) est une adaptation du protocole de Zivy et coll. [12]. Vingt µl d'échantillon ont été déposés sur chaque gel d'IEF de 100 mm de long, 1 mm de diamètre et de composition suivante : 3,78% d'acrylamide, 0,22% N-N' méthylène bis-acrylamide, 2% Triton X-100, 9,2 M urée, 4% Pharmalytes pH 5-8. Tous les gels subissent la même IEF (4000 V/h) avec une solution cathodique (50 mM NaOH) et anodique (50 mM H_3PO_4) et sont équilibrés 15 mn dans le tampon suivant (62,5 mM Tris pH 6,8; 2,3% SDS). Ils sont déposés sur des gels de seconde dimension (concentration constante d'acrylamide à 12%) selon Laemmli [13], L'électrophorèse est conduite à 150 V pendant 1 h 30 mn et révélée selon la méthode de coloration au nitrate d'argent d'Irie et coll. [14].

Pour chacun des deux types morphologiques bien contrastés du greffon (forme juvénile ou forme mature), quatre électrophorégrammes ont été observés essentiellement sur les clones n^{os} 03 et 15, d'origine différente et bien représentatifs de l'état mature [10].

Note présentée par Alexis Moyse.

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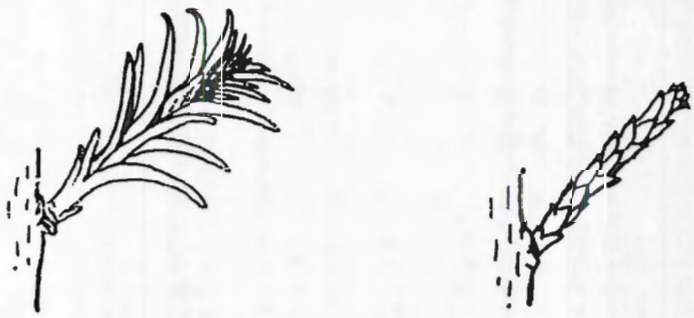

Fig. 1. — Types morphologiques des greffons observés : morphologie rajeunie à gauche, morphologie mature à droite.

Fig. 1. — Scion morphology observed: left, rejuvenated form; right, mature form.

RÉSULTATS ET DISCUSSIONS. — Les électrophorégrammes en bidimension de protéines de méristèmes correspondant au type morphologique juvénile et mature sont présentés sur la figure 2 pour les clones n^{os} 03 et 15.

Quelle que soit l'identité génétique du clone considéré, néanmoins appréciable à travers les profils électrophorétiques observés [15], les greffons à morphologie mature caractérisée sur la figure 1, se différencient de leurs homologues morphologiquement rajeunis par une population protéique globalement plus dense avec une intensification de la présence de protéines acides. Cette tendance a pu être vérifiée quel que soit l'origine et les particularités du profil protéique initial du méristème, reflétant dans l'ensemble l'hétérogénéité notable rencontrée chez un grand nombre de végétaux ligneux et observée par Cross [16] sur le séquoia géant. Cette variabilité entrave considérablement l'analyse des effets spécifiques du microgreffage sur les points végétatifs greffés.

D'autres facteurs perturbateurs doivent également être considérés, tels que l'état physiologique du porte-greffe *in vitro* au moment du greffage ([7], [17]) et la qualité de l'union de la greffe ([4], [18]). Ces éléments interviennent vraisemblablement dans la variabilité intraclonale du matériel greffé [7].

Néanmoins, les différences de densité de la population protéique observée, correspondant à deux expressions morphologiques bien contrastées, incitent à analyser la dynamique

EXPLICATIONS DE LA FIGURE 2

Fig. 2 A. — Électrophorèse bidimensionnelle d'un méristème issu d'un clone centenaire n° 15 et greffé sur un semis *in vitro*. Le microgreffon présentait une morphologie de type mature.

Fig. 2 A. — Two dimensional electrophoresis of one single meristem removed from one hundred year old clone No. 15 *grafted onto young seedling*, in vitro. *Scion morphology was mature form.*

Fig. 2 B. — Électrophorèse bidimensionnelle d'un méristème issu d'un clone centenaire n° 15 et greffé sur un semis in vitro. Le microgreffon présentait une morphologie de type juvénile.

Fig. 2 B. — Two dimensional electrophoresis of one single meristem removed from one hundred year old clone No. 15 *grafted onto young seedling*, in vitro. *Scion morphology was juvenile form.*

Fig. 2 C. — Électrophorèse bidimensionnelle d'un méristème issu d'un clone centenaire n° 03 et greffé sur un semis in vitro. Le microgreffon présentait une morphologie de type mature.

Fig. 2 C. — Two dimensional electrophoresis of one single meristem removed from one hundred year old clone No. 03 *grafted onto young seedling*, in vitro. *Scion morphology was mature form.*

Fig. 2 D. — Électrophorèse bidimensionnelle d'un méristème issu d'un clone centenaire n° 03 et greffé sur un semis in vitro. Le microgreffon présentait une morphologie de type juvénile.

Fig. 2 D. — Two dimensional electrophoresis of one single meristem removed from one hundred year old clone No. 03 *grafted onto young seedling*, in vitro. *Scion morphology was juvenile form.*

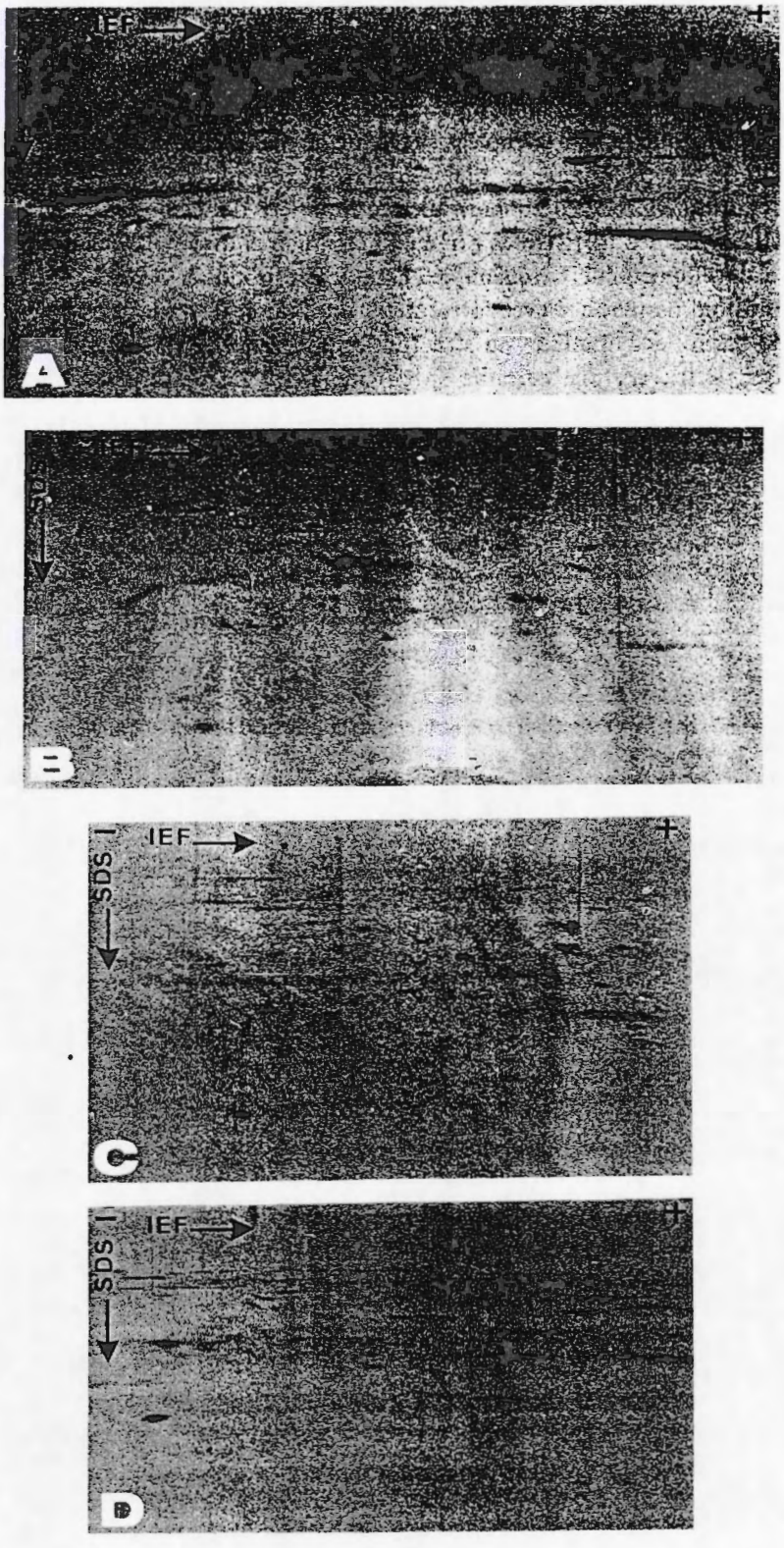

Fig. 2

de la synthèse protéique dans les deux cas en tenant compte du taux de renouvellement des protéines. Ainsi la concentration protéique méristèmatique plus élevée chez le greffon de type mature pourrait s'expliquer par une adaptabilité du métabolisme protéique plus difficile, face aux stimulus physiologiques inhérents au greffage. Des analyses connexes de la synthèse protéique *in vivo* effectuées sur le même matériel supportent ces hypothèses.

D'un point de vue technique et plus général, l'électrophorèse bidimensionnelle des protéines devrait améliorer nos connaissances en matière de vieillissement des arbres, en travaillant à l'échelle des méristèmes primaires caulinaires directement impliqués dans le processus de développement ontogénique. Dans cette optique, les protéines du fait de leur concentration tissulaire élevée présentent un intérêt certain. La diversité de ces composés, de nature enzymatique ou structurale, pourrait être mise à profit pour tenter de caractériser, à travers une certaine spécificité, l'état de maturation des méristèmes considérés. Cette option rationnelle apparaît novatrice pour l'étude biochimique des phénomènes de rajeunissement, en constatant que les méthodes d'investigation proposées jusqu'alors dans ce domaine ne sont pas focalisées sur les points végétatifs, à l'origine des réversions de phase morphologiques constatées.

CONCLUSION. — Les analyses présentées montrent que l'expression morphologique du greffon suite au microgreffage, correspond à des modifications de nature protéique au sein du méristème terminal edificateur. Ce résultat original, basé sur une technique de microanalyse performante, illustre la possibilité d'étudier les mécanismes physiologiques impliqués dans le greffage. Sur ces bases, la poursuite de telles études en privilégiant l'aspect qualitatif de la détection, puis le suivi de polypeptides spécifiques, devraient favoriser la compréhension de certains phénomènes non encore élucidés, tels que l'effet rajeunissant du porte-greffe ([3], [5], [7]).

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Analyses microscopiques de points végétatifs de *Sequoiadendron giganteum* jeunes et âgés durant le repos végétatif et lors du débourrement (*)

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Résumé.- Des analyses microscopiques ont été réalisées dans le but de comparer des points végétatifs de séquoias géants (*Sequoiadendron giganteum*) jeunes et âgés durant le repos végétatif hivernal et lors du débourrement. Pour les deux types de matériels, la reprise de croissance correspond à une augmentation significative de la concentration en ARN cytoplasmiques estimée par cytophotométrie. Les observations cytomorphologiques permettent d'établir des différences sensibles en fonction de l'âge des individus de référence. Lors du débourrement, les divers indices considérés présentent une plus grande similitude avec les caractéristiques des formes juvéniles.

Summary.- Microscopic analyses were achieved in order to compare apices of young and mature giant sequoias (*Sequoiadendron giganteum*) during rest phase and bud-break. For both materials, the beginning of growth was connected to a significant increase of cytoplasmic RNA content estimated cytophotometrically. Cytomorphologic characteristics pointed out noticeable differences related to the age of the donor plant. During bud-break, the various analytical criteria observed tended to the juvenile form characteristics.

Key words : apical meristems - bud-break - cytomorphological analyses - mature - rest period - *Sequoiadendron giganteum* - young.

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INTRODUCTION

L'étude des possibilités de clonage conforme de séquoias géants (*Sequoiadendron giganteum* Buchholz) jeunes et âgés (Monteuuis, 1985) nous a tout naturellement orientés vers les méristèmes apicaux caulinaires, à l'origine des processus de régénération végétative (Nozeran et coll., 1982 ; Hackett, 1985). Les observations relatives à la culture *in vitro* de ces structures minuscules (150 à 200 µm hors tout) révèlent une influence très nette de l'âge de la tête de clone et de

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l'état physiologique au moment du prélèvement sur le comportement ultérieur des explants (Monteuuis, 1987a). Par rapport au repos végétatif hivernal, le débourrement correspond à une stimulation des capacités de clonage des méristèmes excisés et introduits en culture à cette période. Cet effet bénéfique est particulièrement sensible sur les explants provenant des sujets âgés, globalement moins réactifs que leurs homologues issus d'individus juvéniles. Ces constatations, étayées de dosages biochimiques connexes (Monteuuis et Gendraud, 1987), nous ont incités à poursuivre nos analyses au niveau microscopique.

MATERIEL ET METHODES

Origine des échantillons

Les méristèmes primaires caulinaires de *Sequoiadendron giganteum* observés proviennent d'un matériel jeune issu du bouturage d'un semis de 18 mois, et d'un matériel âgé constitué de greffes d'une tête de clone centenaire. Ces deux clones à port bas et diffus, obtenus deux ans auparavant et cultivés depuis lors à l'extérieur à l'Université de Clermont-Ferrand, sont représentatifs des deux classes d'âges condidérées (Monteuuis, 1985). Les extrémités d'axes végétatifs étudiées sont récoltées durant le repos végétatif de janvier, puis lors du débourrement, début mai.

Préparation des échantillons

Les extrémités caulinaires végétatives débarassées de la quasi-totalité de leurs formations foliaires sont fixées dans le mélange : éthanol 95 %-formaldéhyde 40 %-acide acétique glacial (17/2/1, v/v/v), puis déshydratées par une série de bains d'alcool-toluène (Jensen, 1962) avant inclusion dans de la paraffine. Les coupes longitudinales médianes de 7 µm d'épaisseur analysées en microscopie optique ont été colorées par l'Azur B, selon la technique préconisée par Jensen (1962), en vue de l'estimation par cytophotométrie des teneurs en ARN (Riding, 1976 ; Davies, 1984).

Méthodes d'analyses, mesures et traitements des données

Les coupes de matériel jeune et de matériel âgé ont été systématiquement appariées tout au long de la procédure de coloration à l'Azur B. Pour chaque série, nous avons très scrupuleusement suivi les indications de Jensen (1962), notamment en ce qui concerne la durée et la concentration des différents bains de traitement. Les teneurs en ARN cytoplasmiques, après avoir vérifié la spécificité de la réaction à l'Azur B (Jensen, 1962), sont mesurées à l'aide d'un cytophotomètre Reichert pour la longueur d'onde de 665 nm (Jensen, 1962 ; Davies, 1984), valeur retenue à partir de la courbe d'extinction préalablement établie. Dix relevés par méristème observé ont été effectués de façon aléatoire dans des cellules réparties dans le "méristème de flanc" périphérique (Cross, 1943 ; Romberger, 1963 ; Schüepp, 1966), encore appelé "eumériste" (Camefort, 1956).

Les contours des points végétatifs observés, ainsi que des cellules et des noyaux qu'elles renferment, sont reportés au moyen d'une chambre claire et en précisant l'échelle, sur une feuille de papier afin de réaliser les relevés cytomorphométriques correspondants. Les mesures des cellules et noyaux ont été établies essentiellement au moyen d'un traceur d'images Leitz ASM 68 K, en assimilant ces structures à des sphères, modèle de référence le plus satisfaisant (Schüepp, 1966). Les observations se rapportent à un échantillonnage représentatif de l'ensemble de la partie sommitale du dôme méristématique définie par la hauteur h par rapport au méristème médullaire et le diamètre d à ce niveau (Pl. I), après avoir constaté, en accord avec Cross (1943), que les critères cytomorphologiques d'analyse retenus ne justifiaient pas de distinguer une zonation au sein du territoire d'investigation ainsi circonscrit.

L'activité mitotique est exprimée par le nombre moyen de mitoses détectées, par coupe longitudinale médiane de dômes méristématiques, jusqu'au niveau d'apparition du plus jeune primordium observé (Gifford et Corson, 1971).

Les mesures expérimentales sont exprimées sous forme de moyennes rapportées à l'effectif N d'observations effectuées par variante de traitement, et en précisant l'intervalle de confiance établi au seuil $p_0 = 5\%$ (Snedecor et Cochran, 1957).

Les influences respectives des facteurs expérimentaux "âge" -matériel jeune ou âgé- et "stade végétatif" - repos ou débourrement - sur les critères observés présentant le plus d'intérêt, ont été mises en évidence au moyen d'analyses de variance à deux critères de classification en précisant le degré de signification p correspondant (Snedecor et Cochran, 1957).

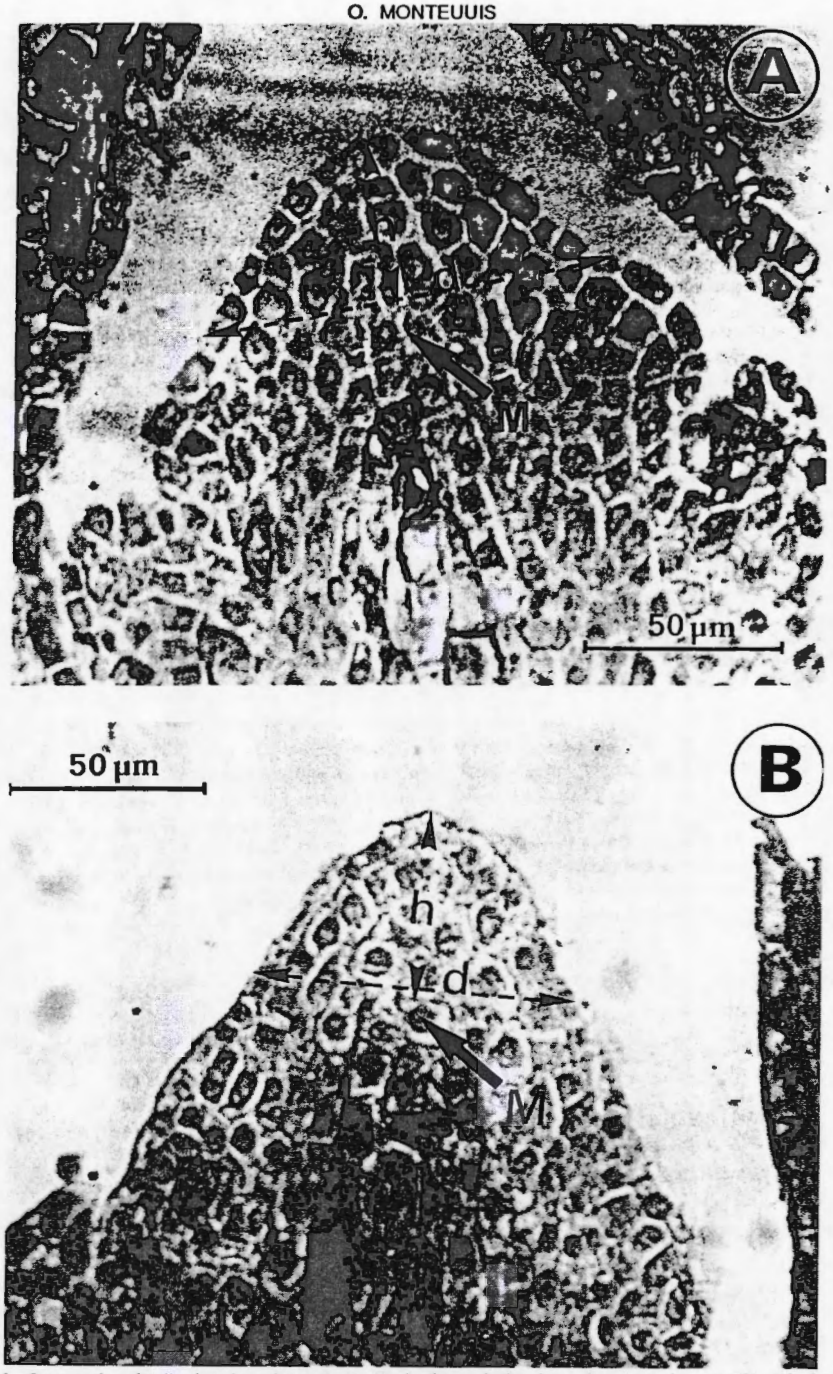


Planche I.- Coupes longitudinales de points végétatifs de *Sequoiadendron giganteum* jeunes (A) et âgés (B) lors du débourrement. d et h, respectivement le diamètre et la hauteur, sont définis à partir du méristème médullaire M.

Plate I.- Median longitudinal sections of shoot apices of young (A) and mature (B) *Sequoiadendron giganteum* at the time of bud-break. d and h, the diameter and the height respectively, are defined from the pith meristem M.

RESULTATS

Les moyennes de densités optiques relatives à l'estimation des teneurs en ARN cytoplasmiques figurent, pour chaque combinaison expérimentale, dans le tableau I.

Tableau I.- Résultats moyens, en densité optique, des teneurs en ARN cytoplasmiques mesurées par cytophotométrie à 665 nm dans le méristème de flanc de points végétatifs de séquoias géants jeunes et âgés durant le repos végétatif et lors du débourrement. Les intervalles de confiance ont été établis au seuil $p_0 = 5$ % pour un effectif observé N = 22.

Table I.- Means of optical density measurements at 665 mm of cytoplasmic RNA contents within the meristem peripheral zone of young and mature *Sequoiadendron giganteum* during the rest period and at the time of bud-break. The confidence intervals were calculated at $p_0 = 0.05$ level for N = 22 measurements per combinaison.

	Sujets jeunes	Sujets âgés
Repos végétatif	0,161 ± 0,038	0, 182 ± 0,047
Débourrement	0,379 ± 0,124	0,362 ± 0,141

Par rapport au repos végétatif, le débourrement correspond à une augmentation très significative ($p < 0,001$) de la concentration des ARN cytoplasmiques et, comme le reflète le tableau II, de l'activité mitotique ($p < 0,001$), essentiellement localisée dans le méristème de flanc. En revanche, aucune influence notable de l'âge de la tête de clone sur ces deux indices n'a pu être décelée.

Tableau II.- Activité mitotique, exprimée par le nombre moyen de mitoses détectées par coupe longitudinale médiane de dôme méristématique jusqu'au niveau d'apparition du plus jeune primordium observé. Les intervalles de confiance ont été établis au seuil $p_c = 5$ % pour un effectif N indiqué.

Table II.- Mitotic activity expressed by the average number of mitoses detected for each median longitudinal section of meristematic dome up to the youngest leaf primordium. The confidence intervals at $p_0 = 0.05$ level and the relevant numbers of observations N are indicated.

	Sujets jeunes	Sujets âgés
Repos végétatif	0 (N = 30)	0 (N = 30)
Débourrement	1,05 ± 0,42 (N = 39)	0,89 ± 0,40 (N = 53)

Les résultats correspondant aux différentes mesures cytomorphologiques, effectuées à partir de l'échantillonnage de coupes longitudinales médianes des points végétatifs, sont récapitulés dans le tableau III. Les volumes des dômes méristématiques ont été établis par calcul intégral à partir des modélisations des contours méristématiques, résultats d'une précédente étude (Monteuuis, 1987b). Ces données ont permis de calculer les nombres moyens de cellules correspondants, à partir des dimensions cellulaires unitaires, et conformément au modèle sphérique adopté.

L'influence des deux facteurs expérimentaux "âge" et "état végétatif" qui apparaît à travers le tableau III pour l'ensembe des indices morphométriques, a été plus particulièrement analysée pour le rapport d/h, le rapport nucléoplasmique, le volume du dôme méristématique et l'effectif cellulaire correspondant. Les résultats sont synthétisés sous forme d'histogrammes dans le tableau IV.

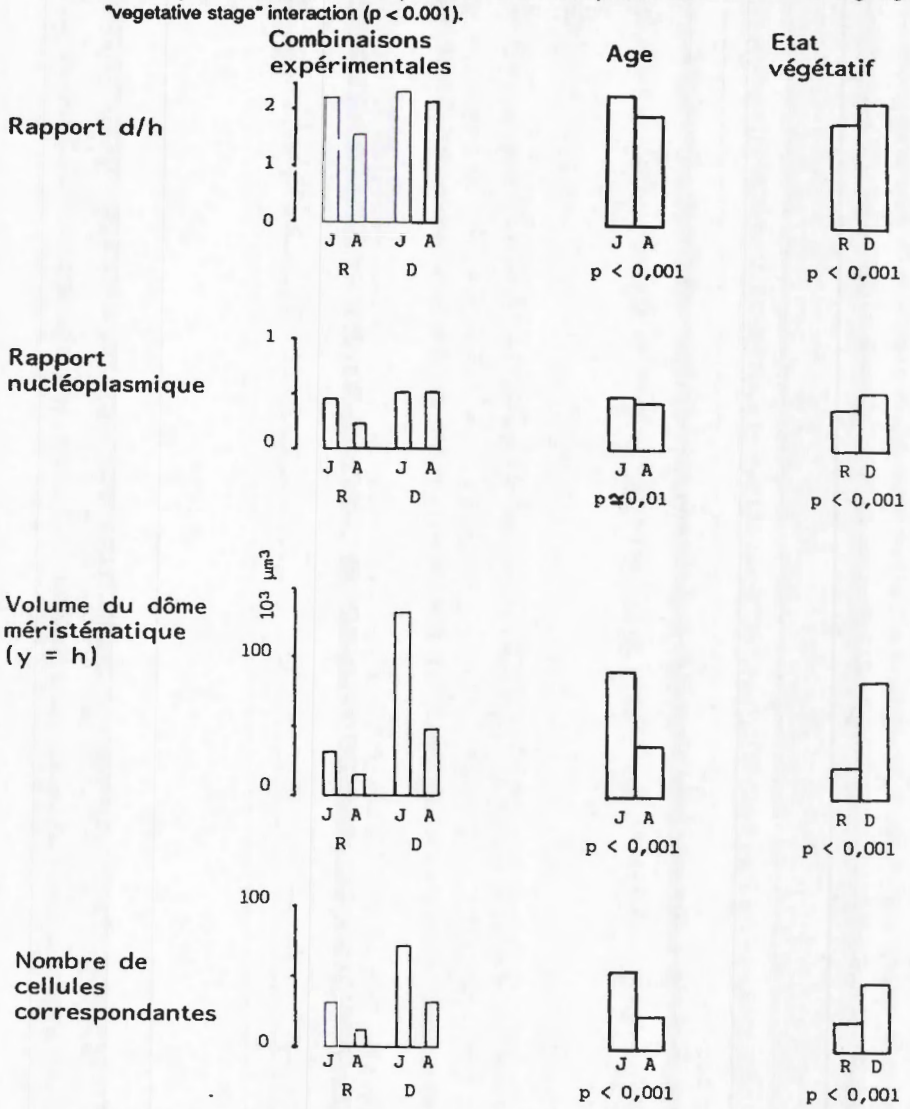
Tableau III.- Données cytomorphologiques moyennes établies à partir de coupes médianes longitudinales de méristèmes primaires caulinaires de séquoias géants jeunes et âgés durant le repos végétatif et lors du débourrement. Les intervalles de confiance précisés ont été calculés au seuil p_0 = 5 % à partir de l'effectif N observé.

Table III.- Means of cytomorphologic measurements established for each median longitudinal section of shoot apices of young and mature giant sequoias observed during the rest period and at the time of bud-break. The confidence intervals at p_0 = 0.05 level and the relevant numbers observations N are indicated.

	REPOS VEGETATIF		DEBOURREMENT	
	sujets jeunes	sujets âgés	sujets jeunes	sujets âgés
Données linéaires en µm				
épaisseur des parois	0,80 ± 0,05 N = 130	0,82 ± 0,05 N = 130	0,93 ± 0,04 N = 320	0,92 ± 0,04 N = 320
rayon cellulaire intérieur	5,41 ± 0,16 N = 76	5,87 ± 0,16 N = 81	6,63 ± 0,15 N = 96	6,23 ± 0,20 N = 96
rayon cellulaire extérieur	6,21 ± 0,21 N = 76	6,69 ± 0,21 N = 81	7,56 ± 0,19 N = 96	7,15 ± 0,24 N = 96
rayon nucléaire	3,41 ± 0,20 N = 76	3,61 ± 0,10 N = 81	4,53 ± 0,12 N = 96	4,20 ± 0,13 N = 96
hauteur h entre le méristème médullaire et le sommet	51,75 ± 2,98 N = 30	57,54 ± 3,79 N = 30	59,65 ± 3,02 N = 39	57,37 ± 3,30 N = 53
diamètre d au niveau du méristème médullaire	115,79 ± 5,96 N = 30	91,40 ± 5,79 N = 30	137,11 ± 5,30 N = 39	125,39 ± 6,49 N = 53
rapport d/h	2,24 ± 0,07 N = 30	1,60 ± 0,08 N = 30	2,35 ± 0,14 N = 39	2,19 ± 0,07 N = 53
équation du contour méristématique (Monteuuis, 1987b)	$y = 0,41\ x^{1,60}$ N = 30	$y = 0,57\ x^{1,80}$ N = 30	$y = 0,41\ x^{1,34}$ N = 30	$y = 0,46\ x^{1,51}$ N = 30
Données en volume en μm^3				
volume cellulaire Vc	698 ± 64 N = 76	891 ± 74 N = 81	1270 ± 85 N = 96	1093 ± 109 N = 96
volume nucléaire Vn	207 ± 19 N = 76	205 ± 17 N = 81	409 ± 32 N = 96	332 ± 32 N = 96
volume cellulaire, paroi comprise	1003 ± 105 N = 76	1254 ± 123 N = 81	1810 ± 140 N = 96	1531 ± 159 N = 96
rapport nucléoplasmique Vn/(Vc - Vn)	0,46 ± 0,04 N = 76	0,32 ± 0,03 N = 81	0,52 ± 0,05 N = 96	0,52 ± 0,06 N = 96
équation du volume du dôme méristématique	$V = 4,25\ y^{2,25}$	$V = 2,78\ y^{2,11}$	$V = 4,77\ y^{2,49}$	$V = 3,78\ y^{2,32}$
Volume du dôme méristématique pour y = h	$[31,4 \pm 3,6] \times 10^3$ N = 30	$[14,6 \pm 1,9] \times 10^3$ N = 30	$[131,1 \pm 15,0] \times 10^3$ N = 39	$[48,7 \pm 5,9] \times 10^3$ N = 53
nombre de cellules correspondant (y = h)	31,4 ± 3,6	11,8 ± 1,5	72,4 ± 8,3	31,8 ± 3,9

Tableau IV.- Influence des deux facteurs expérimentaux "âge" - jeune (J) et âgé (A) - et "état végétatif" - repos (R) et débourrement (D) - sur le rapport d/h, le rapport nucléoplasmique, le volume du dôme méristématique et le nombre de cellules correspondant. p indique le degré de signification résultant des analyses de variance appliquées qui font ressortir, pour chacun des critères considérés, une très forte interaction "âge" x "état végétatif" ($p < 0,001$).

Table IV.- Influence of the two experimental factors : "age" - young (J) and mature (A) - and "vegetative stage" - rest (R) and bud-break (D) - on the d/h ratio, the nucleoplasmic ratio, the meristematic dome volume and the relevant numbers of included cells. p indicates the significant range resulting from the analyses of variance, which point out for the four parameters observed a strong "age" x "vegetative stage" interaction ($p < 0.001$).



L'effet de l'âge des pieds-mères d'origine se ressent pour l'ensemble des quatre critères examinés ($p < 0,01$), surtout en ce qui concerne les caractéristiques des dômes méristématiques - rapport d/h, volume, effectif cellulaire - ($p < 0,001$), les valeurs les plus élevées correspondant au matériel jeune.

L'influence de l'état végétatif apparaît de façon très significative ($p < 0,001$) indifféremment pour chacun de ces quatre mêmes critères. Le débourrement correspond à une augmentation très nette des valeurs obtenues durant le repos végétatif, qui avoisinent alors les résultats caractérisant le matériel jeune.

Consécutivement aux analyses de variance appliquées, la mise en évidence d'une très forte interaction âge x état végétatif ($p < 0,001$) incite à considérer les résultats relatifs à chaque combinaison expérimentale.

En ce qui concerne le rapport nucléoplasmique, il est intéressant de noter que la différence entre le matériel jeune et le matériel âgé, très prononcée durant le repos végétatif, s'estompe au stade débourrement. Cette tendance s'applique, mais dans une moindre mesure, au rapport d/h qui préfigure malgré tout que si le débourrement est associé à un accroissement des résultats obtenus durant le repos végétatif, les valeurs élevées peuvent demeurer l'apanage du matériel jeune. Ceci est pleinement vérifié dans le cas du volume du dôme méristématique et de son effectif cellulaire, où les différences entre matériel jeune et âgé persistent de façon très marquée, que ce soit durant le repos végétatif ou le débourrement.

DISCUSSION

A l'instar d'autres Gymnospermes (Camefort, 1954 ; Gifford et Corson, 1971 ; Owens et Molder, 1973), la reprise de croissance dans les méristèmes caulinaires végétatifs de *Sequoiadendron giganteum* s'accompagne d'une nette augmentation, par rapport au repos végétatif, des ARN, essentiellement localisés dans le cytoplasme des cellules de méristème de flanc (Camefort, 1954, 1956 ; Riding et Gifford, 1973 ; Riding, 1976). Cet accroissement des ARN et de l'activité mitotique sous l'effet d'agents extérieurs plus favorables correspond vraisemblablement à une stimulation générale du métabolisme, comme l'illustrent les dosages biochimiques d'acides nucléiques, de nucléotides et les analyses des systèmes traductionnels effectués sur les mêmes échantillons (Monteuuis et Gendraud, 1987 ; Bon, 1988).

Conjointement, les méristèmes prélevés à cette période en vue du clonage expriment en culture *in vitro* des facultés organogènes bien supérieures à leurs homologues excisés de bourgeons en repos végétatif hivernal (Monteuuis, 1987a). Plusieurs travaux (Trippi et Brulfert, 1973 ; Riding, 1976 ; Davies, 1984) ont permis d'établir, de façon similaire, que l'aptitude à la multiplication végétative était d'autant plus développée que les teneurs en ARN des méristèmes caulinaires étaient élevées.

Des analyses antérieures ont montré une influence significative du stade végétatif et de l'âge de la tête de clone sur la conformation des dômes méristématiques (Monteuuis, 1987b), plus volumineux pour le matériel jeune et lors du débourrement, comparativement au matériel âgé et au repos végétatif. Cette tendance s'exprime de façon plus immédiate par les valeurs du quotient d/h, inspiré des travaux de Tepper (1963), Owston (1969) et Riding (1976) que nos résultats confirment, et défini à partir du méristème médullaire (voir planche I). Signalons à ce propos, l'intérêt de cette référence anatomique judicieuse qui permet, pour certaines analyses, de circonscrire sans ambiguïté le champ d'investigations au sommet du méristème, secteur déterminant du point de vue ontogéni-

que (Cross, 1943 ; Camefort, 1956 ; Gifford et Corson, 1971). Les effets des deux facteurs expérimentaux étudiés s'illustrent également par les modifications des indices cytomorphologiques infra-méristématiques établis, conformes aux valeurs communiquées par Cross (1943). Ainsi, il est intéressant de remarquer, entre autres, que les cellules du clone jeune deviennent plus grandes que celles du clone âgé lors de la reprise de croissance, confirmant alors les observations de Stein et Fosket (1969) sur des extrémités caulinaires végétatives d'*Hedera helix* poussant. L'ensemble de ces divers éléments structuraux permet d'établir que les points végétatifs de jeunes séquoias géants sont plus volumineux et renferment plus de cellules que leurs homologues issus d'individus âgés. Ce résultat, vérifié aussi bien durant le repos végétatif que lors du débourrement, singularise notre matériel d'étude par rapport à d'autres, non arborescents et cultivés *in vitro* dans un environnement très particulier il est vrai (Watelet-Gonod et Favre, 1981). Des observations et des analyses connexes (Monteuuis, 1987a) suggèrent que l'aptitude au clonage *in vitro* de points végétatifs de *Sequoiadendron giganteum* pourrait être positivement corrélée à l'effectif cellulaire du dôme méristématique.

Le bilan synthétique de l'ensemble des analyses présentées confère au facteur "âge", et surtout au facteur "état végétatif", une influence primordiale vis-à-vis des critères microscopiques observés. Plus précisément, le stade débourrement correspond à un accroissement très net des mesures établies au moment du repos, qui tendent alors à atteindre les valeurs caractéristiques du matériel jeune, comme le reflètent notamment les histogrammes du tableau IV. Ces remarques qui corroborent les analyses biochimiques et les observations de culture *in vitro* réalisées sur le même matériel (Monteuuis, 1987a ; Monteuuis et Gendraud, 1987), constituent un argument supplémentaire en faveur de l'interprétation du vieillissement cyclique réitéré des méristèmes primaires au cours de la croissance des unités successives de morphogenèse (Krenke, 1940 ; Franclet, 1983).

CONCLUSION

La présente étude révèle de réelles différences cytomorphologiques au sein de points végétatifs caulinaires de séquoias géants, liées à l'âge des individus. Ce résultat atteste que le phénomène de maturation des végétaux ligneux peut se manifester jusque dans les méristèmes primaires, et confirme par là même les présomptions basées sur des dissemblances remarquées pour plusieurs espèces arborescentes en culture *in vitro*, en fonction de l'âge de la tête de clone (Bekkaoui et coll., 1985 ; Monteuuis, 1987a).

D'un point de vue théorique, les points végétatifs peuvent susciter beaucoup d'espoirs pour le clonage conforme d'arbres âgés en raison des concepts de juvénilité et de totipotentialité associés à la notion même de méristème (Margara, 1982). Il semble que la situation réelle soit plus nuancée à travers la prise en considération, en fonction de l'âge des sujets de référence, de l'influence de l'état physiologique, appréhendée sur notre matériel à partir de stades phénologiques très contrastés - repos végétatif ou débourrement. L'importance de ce paramètre prend toute sa signification si, comme le prétendent Buvat (1955) et Romberger (1963), le devenir organogène des méristèmes demeure étroitement dépendant de leur contexte physiologique. Plusieurs observations nous ont permis de vérifier le bien-fondé de cette opinion qui incite à considérer l'analyse de potentialités

ontogéniques des méristèmes primaires en tenant compte de l'aspect évolutif de leurs caractéristiques en fonction du temps et de l'environnement extérieur, ainsi que de leur situation topologique au sein du végétal (influence des systèmes corrélatifs). Ces arguments justifient, en matière de clonage, les opérations de conditionnement physiologique des pieds-mères, tout en tirant profit de la variabilité constatée pour sélectionner les structures végétatives - points végétatifs, territoires méristématiques, voire cellules - les plus juvéniles, et par là même, les plus prometteuses.

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Analyse cytophotométrique comparée des parois de mésophylle de feuilles de *Sequoiadendron giganteum* jeunes et âgés (*)

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Résumé.- Une technique d'analyse histochimique de composés pariétaux par cytophotométrie a été appliquée à des coupes transversales de feuilles de séquoias géants (*Sequoiadendron giganteum*) jeunes et âgés. Les parois du mésophylle du matériel âgé contiennent plus de polysaccharides que celles du matériel jeune. Cette tendance, qui persiste après l'hydrolyse des substances pectiques, puis des hémicelluloses,et enfin des autres polysaccharides non cellulosiques, serait due, du moins en partie, à des teneurs plus élevées en hémicellulose et en cellulose.

Summary.- A histochemical technique to investigate cytophotometrically cell wall components was applied to transverse sections of leaves belonging to young and mature giant sequoias (*Sequoiadendron giganteum*). Mesophyll cell walls of mature material contained more polysaccharides than the young material ones. This tendency which remained unchanged after the hydrolysis of the pectic substances, then the hemicelluloses and finally the other non cellulosic polysaccharides, could be partly due to higher hemicellulose and cellulose concentrations in the mature material.

Key words : cell wall components - cytophotometric analysis - mesophyll - polysaccharides - young and mature *Sequoiadendron giganteum*.

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INTRODUCTION

Le vieillissement des végétaux ligneux au cours de leur développement est lié à une modification de certaines caractéristiques des formes juvéniles, résumées notamment par Franclet (1983). Cette revue de synthèse fait abondamment référence à plusieurs aspects de la biologie végétale affectés par le phénomène, mais révèle un manque d'informations du point de vue histologique.

Sur notre matériel d'étude, *Sequoiadendron giganteum*, le dimorphisme foliaire marqué en fonction de l'âge (Monteuuis, 1985) nous a incités à

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compléter nos observations morphologiques par des analyses infrastructurales. En nous inspirant d'études histochimiques antérieures (Jensen, 1962 ; Jona et Foa, 1977), nous avons cherché à apprécier par cytophotométrie les effets comparés de divers traitements hydrolytiques sur les parois du mésophylle des feuilles de séquoias géants jeunes et âgés.

MATERIEL ET METHODES

Origine des échantillons

Les échantillons ont été récoltés dans les mêmes conditions sur des pieds-mères clonés obtenus 3 ans auparavant, en distinguant un clone jeune issu du bouturage d'un semis de séquoia géant de 18 mois, d'un clone âgé provenant d'un séquoia géant centenaire greffé (Monteuuis, 1985). Ce matériel, au port bas et diffus entretenu par des prélèvements fréquents, est cultivé à l'extérieur à l'Université de Clermont-Ferrand. Les feuilles analysées sont prélevées dans la partie médiane de la pousse de l'année, durant le repos hivernal des pieds-mères.

Préparation des échantillons

Les feuilles récoltées sont fixées dans un mélange : éthanol 95 %, formaldéhyde 40 %, acide acétique glacial (17/2/1 ; v/v/v), puis déshydratées avant inclusion dans la paraffine (Jensen, 1962). La partie libre de chaque feuille en alène (Monteuuis, 1985) est débitée transversalement au microtome en sections de 10 µm d'épaisseur, réparties ensuite équitablement sur 4 lames porte-objet pour constituer un lot (Jensen, 1960 et 1962). Chaque lot de matériel âgé est systématiquement apparié à un lot de matériel jeune pour être soumis, après déparaffinage, à la succession de traitements hydrolytiques détaillés ci-après, en conservant à l'issue de chaque étape une lame pour analyse (Jensen, 1962) : A - aucun traitement témoin ; B - immersion durant 12 heures à 30°C dans une solution aqueuse de pectinases (EC 3.2.1.15., Sigma) à 7000 unités/l, puis trempage pendant 2 heures à 90°C dans une solution à 0,5 % d'oxalate d'ammonium ; C - trempage durant 12 heures à température ambiante dans une solution à 4 % de NaOH ; D - trempage durant 12 heures à température ambiante dans une solution à 17,5 % de NaOH.

Ces différentes étapes sont destinées à éliminer successivement et respectivement les substances pectiques (B), les hémicelluloses (C) et les autres polysaccharides non cellulosiques pour ne conserver théoriquement que la cellulose (D) (Jensen, 1960 ; Jona et Foa, 1977). Après chaque traitement, les lames sont rincées abondamment et avec précaution dans de l'eau distillée. Le décollement des coupes, véritable handicap, a été limité par un film d'éthyle cellulose (Sigma) - 0,5 % dans un mélange de toluène/alcool absolu (4/1 ; v/v) qui doit être renouvelé à chaque traitement chimique (Jona et Foa, 1977 ; Jona et coll., 1981). A l'issue de ces hydrolyses, les lots de matériel âgé et de matériel jeune appariés sont colorés au P.A.S. (acide périodique-Schiff) conformément à la procédure préconisée par Jensen (1962).

Mesures, expression et traitements des données

Les effets des différents traitements hydrolytiques ont été analysés quantitativement au niveau des parois du mésophylle à l'aide d'un cytophotomètre Reichert. La surface effective photométrée à travers les parois de 1,2 µm d'épaisseur moyenne correspond à un disque de 1 µm de diamètre. L'intensité de la coloration, proportionnelle à la teneur en polysaccharides, est indiquée par la transmission T (Jona et Foa, 1977 ; Jona et coll., 1981) mesurée au moyen d'un photomultiplicateur pour la longueur d'onde de 560 nm, valeur retenue après établissement de la courbe d'extinction (Lison, 1960). Les résultats analysés sont exprimés en densité optique (D.O.) définie par la relation : D.O. = - log T (Lison, 1960), et correspondant aux moyennes établies à partir des 30 mesures par coupe pour chacune des 4 lames des lots. Le test statistique de Wilcoxon (Snedecor et Cochran, 1957) a été appliqué pour comparer les échantillons du clone jeune à ceux du clone âgé, au sein des lots constamment appariés au cours des différentes séquences d'hydrolyses, puis de coloration au P.S.A. Les comparaisons 2 à 2 ont été effectuées sur 10 à 18 paires de coupes par variante de traitement.

RESULTATS

Une analyse de variance appliquée à l'ensemble des lots traités pour chacun des 2 clones indique un effet significatif ($p < 0,001$) des différents traitements hydrolytiques sur les valeurs mesurées par cytophotométrie, et ce, malgré une variabilité intra-classe certaine, exprimée par l'amplitude des intervalles de confiance ($p = 5$ %) de la figure.

Les comparaisons inter-clonales réalisées à partir des échantillons

appariés traduisent une teneur pariétale en polysaccharides totaux plus élevée dans le mésophylle des feuilles du matériel âgé que dans celui du matériel jeune ($0,001 < p < 0,005$). Cette tendance persiste après l'hydrolyse des substances pectiques ($0,001 < p < 0,005$), puis des hémicelluloses ($0,001 < p < 0,005$), et enfin des autres polysaccharides non cellulosiques ($0,01 < p < 0,025$). Pour chacun des 2 clones, les moyennes correspondant à l'ensemble des lots analysés par variante de traitement sont illustrées dans la figure 1.

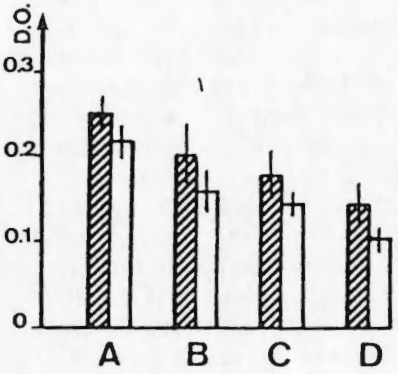

Fig. 1.- Moyennes des résultats cytophotométriques - en densité optique D.O. - par variante de traitement et par clone, de l'ensemble des lots appariés comparés ; les intervalles de confiance sont représentés pour p = 5 %.
A : aucune hydrolyse (témoin) ; B : après hydrolyse des substances pectiques ; C : après hydrolyse des substances pectiques et des hémicelluloses ; D : après hydrolyse des substances pectiques, des hémicelluloses et des autres polysaccharides non cellulosiques. ▭ clone jeune. ▨ clone âgé.

Fig. 1.- Extinction value averages according to the different experimental treatments and to the clone. Vertical bars represent confidende intervals (p = 5 %).
A : control ; B : after removal of pectic substances ; C : after removal of pectic substances and hemicelluloses ; D : after removal of pectic substances, hemicelluloses and other non cellulosic polysaccharides. ▭ young clone. ▨ mature clone.

L'analyse comparative inter-clonale, en considérant individuellement chaque catégorie de composés pariétaux d'après leur nature (composés pectiques, hémicelluloses, autres polysaccharides non cellulosiques et finalement la cellulose - Jensen, 1960 et 1962 ; Jonas et coll., 1981), n'indique pas de différence significative, hormis pour les hémicelluloses et la cellulose, plus abondantes dans le vieux matériel (respectivement $0,025 < p < 0,05$ et $0,01 < p < 0,025$).

DISCUSSION

La technique d'histochimie associée aux mesures cytophotométriques présentée permet de comparer des structures microscopiques visualisées bien précises avec une bonne sensibilité. Certains aspects méritent néanmoins d'être commentés. La réelle efficacité des différents traitements hydrolytiques spécifiques est difficilement contrôlable. En effet, il est permis de supposer que les hydrolyses des divers composés pariétaux envisagés ne sont pas totales (Roland, 1980 ; Tollier et Riquet, 1980), bien que les traitements décrits, surtout chimiques, soient suffisamment violents pour altérer l'intégrité structurale des coupes et favoriser leur décollement des lames porte-objet, en dépit du film protecteur d'éthyle cellulose, conformément aux remarques d'autres auteurs (Jensen, 1960 et 1962 ; Jona et Foa, 1977 ; Jona et coll., 1981).

La mise en évidence, au niveau du mésophylle de feuilles de séquoias géants, d'une concentration en polysaccharides pariétaux d'autant plus élevée que le sujet est âgé, est un résultat nouveau qui n'en demeure pas moins cohérent. Les différences observées, notamment du point de vue des hémicelluloses et celluloses (voir Jensen, 1960 ; Joseleau, 1980 ; Lorences et coll., 1987a et b), pourraient s'instaurer au cours des processus de différenciation pariétale (Catesson, 1980 ; Gross, 1984) associés au développement des feuilles qui, chez *Sequoiadendron*

giganteum, présentent des dissemblances morphologiques très contrastées en fonction de l'âge des individus (Monteuuis, 1985).

Des analyses similaires connexes, mais quantitativement plus restreintes, effectuées au niveau des parois du parenchyme sous-jacent aux méristèmes apicaux caulinaires, incitent à penser que ces différences de composition pariétale entre jeunes et vieux sujets surviennent précocement au cours de l'édification des structures végétatives aériennes (Lorences et Zarra, 1986 ; Lorences et coll., 1987a et b). Il serait intéressant de poursuivre ces comparaisons au niveau de la chronologie phyllogénique (initium, primordium, ébauche foliaire, jeunes feuilles ...), en considérant également d'autres tissus. Dans le cas présenté de feuilles adultes, seul le mésophylle a répondu favorablement à la technique d'histochimie appliquée, comme nous avons pu le vérifier par les analyses de variance. Les tentatives d'analyses effectuées sur d'autres tissus nous ont amenés à des résultats incohérents, notamment au niveau de l'assise de cellules sous-épidermiques qui possèdent des parois bien plus développées dans le matériel âgé que dans le matériel jeune (240 μm^2 contre 152 μm^2 de section moyenne par coupe cellulaire). Ces déboires pourraient s'expliquer par l'existence d'une matrice de lignines (Jensen, 1960 et 1962 ; Monties, 1980) - composés également réactifs au P.A.S. (Jensen, 1962) - intimement associée aux polysaccharides pariétaux (Jensen, 1962 ; Catesson, 1980 ; Roland, 1980 ; Donaldson, 1985).

CONCLUSION

La technique d'analyse cytophotométrique appliquée au mésophylle de feuilles de *Sequoiadendron giganteum* a permis de mettre en évidence des différences significatives de composition en polysaccharides pariétaux entre jeunes et vieux individus. Ce résultat original mériterait d'être vérifié sur d'autres espèces, et approfondi dans le cadre de l'étude des phénomènes liés au vieillissement des végétaux au cours de leur développement ontogénique.

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Physiologie végétale/*Plant Physiology*

Microgreffage de méristèmes primaires caulinaires de Pins maritimes (*Pinus pinaster* Ait.) âgés sur de jeunes semis cultivés *in vitro*

Élisabeth DUMAS, André FRANCLET et Olivier MONTEUUIS

Résumé — La technique de microgreffage présentée permet de greffer des points végétatifs de *Pinus pinaster* âgés sur de jeunes semis *in vitro* avec un taux de réussite moyen de 68 %. Les méristèmes greffés évoluent plus ou moins rapidement en pousses feuillées, certaines présentant une morphologie juvénile caractérisée qui persiste après acclimatation.

Apical meristem micrografting of mature maritime pines (*Pinus pinaster* Ait.) onto *in vitro* young seedlings

Abstract — The micrografting technique described makes it possible to graft apical meristems belonging to mature Pinus pinaster *onto* in vitro *young seedlings with an average rate of success of* 68%. *The grafted meristems produce more or less rapidly leafy shoots some of which exhibit characterized juvenile morphological traits maintained after acclimatization.*

Abridged English Version — *In vitro* culture of apical meristems excised from mature selected trees for true-to-type cloning purposes must be objectively considered as a very promising technique ([1], [2]). The possibility of removing the meristems from the inhibiting influence of the maturation process affecting the mother plant would enhance their organogenic response in well adapted culture conditions ([3], [4]). At the same time, contamination risks, known to be very damaging in certain conditions, would diminish.

Referring to the studies on micropropagation of *Pinus pinaster*, the above two aspects seem of primordial interest ([5], [6], [7]). Nevertheless, difficulties encountered in excised meristem cultures on synthetic gelified media, especially for mature material [8], incited us to experiment meristem micrografting techniques ([9], [10], [11]) and to take advantage conjointly of the beneficial effect of the rootstock susceptible to induce rejuvenation ([12] to [17]). Given the satisfactory results, it is of interest to describe the micrografting method applied to *Pinus pinaster*.

The seedlings used as rootstocks were obtained from germinated seeds, transferred after sterilization onto a cellulose acetate "Sorbarod" container satured with sterile demineralized water. When the epicotyl reached a height about 1 cm, the seedlings could be grafted.

Terminal parts of auxiblasts with quiescent scaly buds were removed from 11, 80 and 100-year-old maritime pine genotypes. These buds were surface desinfected then rapidly and carefully dissected to clear the apical meristem and its surrounding foliar primordia which stood for the scion and did not exceed 400 µm in length. At this stage, the rootstock with its linked Sorbarod container was pulled out of the culture tube to be superficially incised on its epicotyl side just above the cotyledons (*fig.* 1). The scion was then quickly removed and immediately placed onto the slash. The exudation which flowed out of the incised tissues favored the early adherence of the scion. The next step was to transfer the grafted rootstock onto another Sorbarod saturated with 5 ml of the convenient liquid medium composed of Margara's macronutrients [18], Murashige and Skoog's micronutrients [19], 20 $g.l^{-1}$ sucrose and 20 $g.l^{-1}$ activated charcoal.

Note présentée par Alexis MOYSE.

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This technique was tested on a total of 186 micrografts corresponding to four different aged genotypes with an average success of 68%. Although the experimental design was too restricted to identify any age effect on the survival rates, it appeared that the meristems removed from the youngest material (11.-year old) responded earlier to develop a leafy shoot (*fig.* 2A, B and C) with higher frequency of rejuvenated forms exhibiting euphylls exclusively (*fig.* 2D). In contrast, scions removed from old genotypes were susceptible to remaining quiescent for several weeks before expanding. A great heterogeneity of responses could nevertheless be observed within each genotype (Tab.). Shoot expansion of the leafy scions was stimulated by cutting back the stock above the graft union and removing the lateral shoots arising from the epicotyl. 80 to 100% of the micrografted stocks were successfully acclimatized in the greenhouse taking advantage of the quality of the root system developed in Sorbarod.

This method of meristem micrografting applied to *Pinus pinaster* appeared to be very helpful to promote micropropagation possibilities of mature trees. Such a technique benefited simultaenously from the meristem culture advantages and from grafting onto juvenile stock and proved to be efficient to rejuvenate in certain occasions. From a technical point of view, the beneficial influence of using Sorbarod as physical *in vitro* culture support ought to be emphasized since it facilitated micrografting manipulations with the possibility to graft outside of the tube without damaging the stock root system and later made the acclimatization phase easier. In addition, the exudation secretion from the incised tissues greatly simplified the micrografting procedure by comparison with *Sequoiadendron giganteum* ([10], [11]). . In fact, the connection between the scion and the stock occurred in a very natural way without any exogenous growth regulator or other technical artifice proved to be efficient in other cases ([20], [21]).

These different arguments incited us to develop this micrografting technique making it possible to vegetatively propagate maritime pines from excised meristems with much higher success rates than were to be expected when considering the results obtained up to now. Furthermore, this method seemed in principle applicable to other pine species to improve capacities for true-to-type cloning of selected material as expected by a large number of geneticists, silviculturists, and of course tree propagators.

INTRODUCTION. — La culture *in vitro* de méristèmes primaires caulinaires est une technique très prometteuse, notamment en vue du clonage conforme d'arbres sélectionnés âgés ([1], [2]). La possibilité de soustraire les points végétatifs de l'influence inhibitrice liée au vieillissement de la plante mère favoriserait leur réactivation organogène dans des conditions de culture adéquates ([3], [4]). Conjointement, les risques de contamination sont minimisés.

Dans le cadre des travaux sur la micropropagation du pin maritime (*Pinus pinaster* Ait.), ces deux atouts majeurs prennent toute leur importance eu égard aux problèmes rencontrés ([5], [6], [7]). Néanmoins, les difficultés inhérentes à la culture de méristèmes de cette espèce sur milieux synthétiques, particulièrement dans le cas de matériel âgé [8], nous ont incités à nous orienter vers le microgreffage de points végétatifs ([9], [10], [11]). De plus, cette technique permet simultanément de tirer profit de l'influence bénéfique du porte-greffe, susceptible d'induire un certain rajeunissement du matériel microgreffé ([12] à [17]). Les résultats satisfaisants obtenus sur *Pinus pinaster* nous ont encouragés à décrire la méthodologie de microgreffage de méristèmes mise au point sur cette espèce.

MATÉRIEL ET MÉTHODES. — 1. *Obtention des porte-greffes.* — Les graines de pin maritime, conservées en chambre froide (+4°C), sont désinfectées par trempage dans une solution de péroxyde d'hydrogène à 110 volumes pendant 20 mn, puis abondamment rincées dans trois bains d'eau déminéralisée stérilisée. Les graines sont ensuite ensemencées individuellement en conditions aseptiques dans des tubes de culture droits de 20 × 250 mm coiffés de façon non hermétique de capuchons en matière plastique translucide. Chaque tube contient une motte cylindrique de 20 × 30 mm en acétate de cellulose (« Sorbarod ») imbibée de 5 ml d'eau déminéralisée stérilisée. L'ensemble est ensuite disposé en chambre de culture où des tubes fluorescents « Sylvania Grolux » fournissent une intensité lumineuse de 120 $\mu mol.m^{-2}.s^{-1}$ pendant 16 h; la température est fixée à 25 ± 1°C. Dans ces conditions d'environnement, les graines germent rapidement. Les porte-greffes sont greffés après deux mois de culture, lorsque l'épicotyle atteint 1 cm de hauteur.

2. *Origine des greffons.* — Les greffons sont prélevés à l'extrémité des auxiblastes, au sein de bourgeons écailleux appartenant à quatre génotypes âgés de 11, 80 et 100 ans.

3. *Technique de microgreffage.* — Le microgreffage est réalisé en conditions aseptiques sous loupe binoculaire équipée d'une source de lumière froide.

Après 20 mn de désinfection dans une solution aqueuse d'hypochlorite de calcium à 9 % additionnée de quelques gouttes de mouillant « Teepol », les bourgeons écailleux sont rincés dans trois bains d'eau déminéralisée stérilisée puis rapidement et précautionneusement disséqués afin de dégager le dôme du méristème apical caulinaire entouré de ses primordia. Cet ensemble qui n'excède pas 0,4 mm de hauteur constitue le greffon. A ce stade, le porte-greffe solidaire de son support de culture Sorbarod est sorti du tube de culture, puis entaillé superficiellement sur 2 mm de longueur au niveau de l'axe épicotylé,

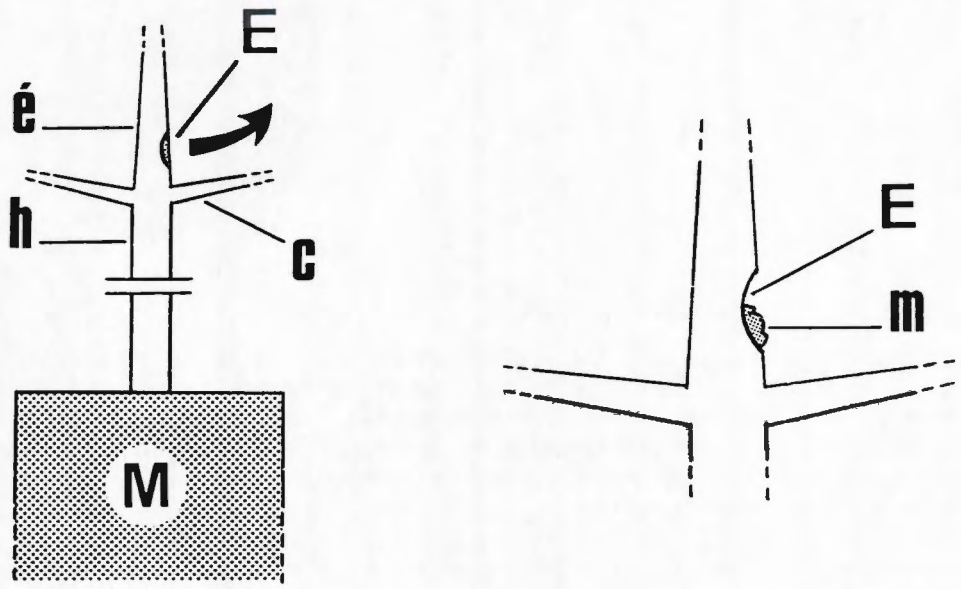

Fig. 1. — Illustration schématisée de la technique de microgreffage décrite : (c) cotylédons du semis porte-greffe; (é) épicotyle; (h) hypocotyle; (m) méristème greffé; (E) entaille superficielle latérale; (M) motte de culture (« Sorbarod »).

Fig. 1. — Schematic illustration of the described micrografting technique: (c) cotyledons of the seedling used as rootstock; (e) epicotyl; (h) hypocotyl; (m) meristem grafted; (E) superficial lateral slash; (M) in vitro *culture container ("Sorbarod").*

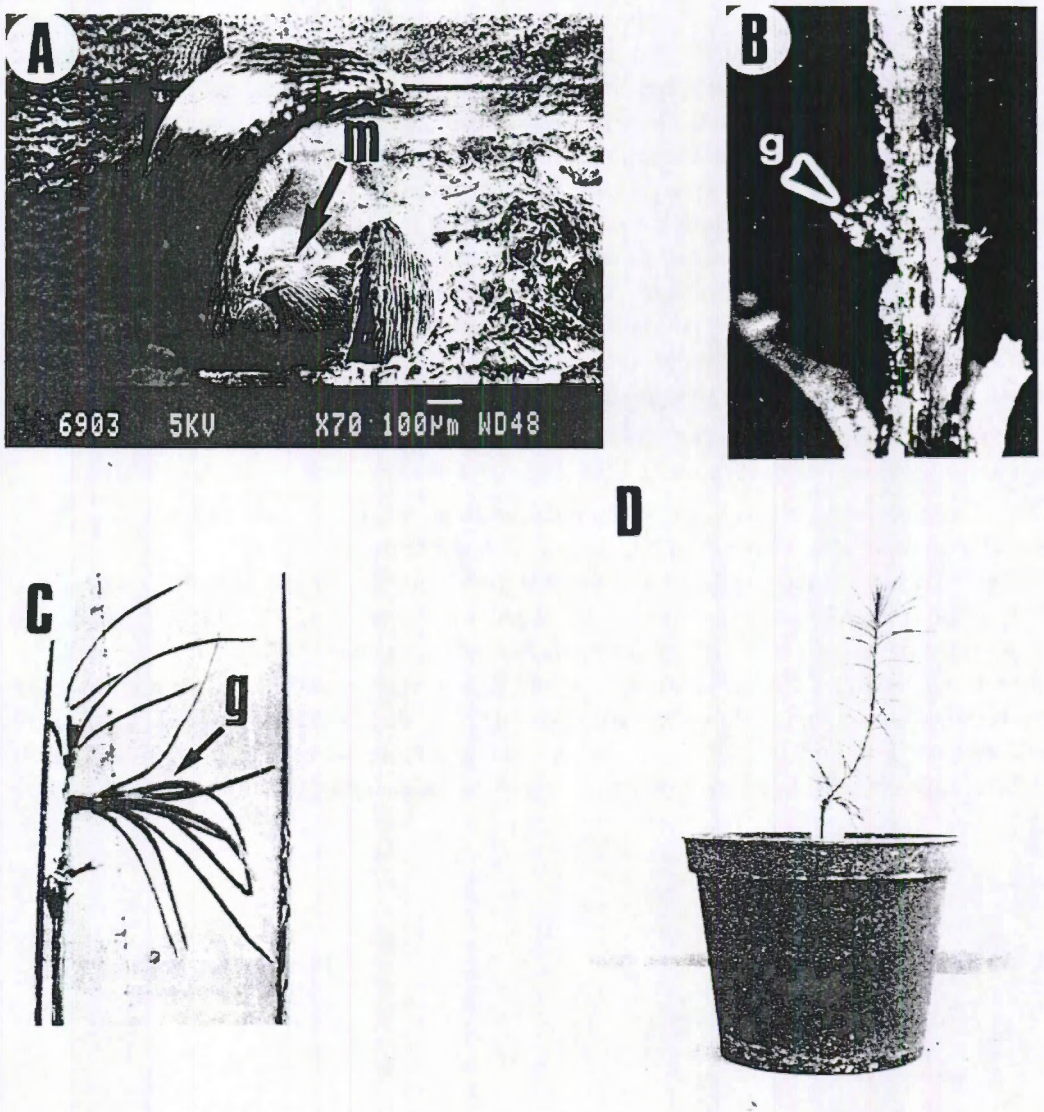

Fig. 2. — Étapes chronologiques du développement du greffon. A, premières phases d'organogenèse du méristème greffé (m); B et C, croissance du greffon (g) sur le côté de l'épicotyle du porte-greffe; D, microgreffe acclimatée présentant des euphylles caractéristiques de l'état juvénile.

Fig. 2. — Chronological stages of the scion development. A, first stages of organogenesis of the grafted meristem (m); B and C, growth of the scion (g) on the side of the rootstock epicotyl; D, acclimatized micrograft exhibiting euphylls which characterize the juvenile state.

à proximité des cotylédons (*fig.* 1). Le greffon est alors rapidement prélevé, par sectionnement basal au moyen d'un éclat de lame de rasoir pour être immédiatement et délicatement déposé sur l'entaille du porte-greffe. L'exsudat qui s'écoule des tissus incisés favorise l'adhérence précoce du greffon. Le microgreffage proprement dit étant terminé, les porte-greffes sont transférés sur un nouveau Sorbarod imbibé de 5 ml du milieu de culture composé des macro-éléments de Margara [18], des micro-éléments de Murashige et Skoog [19], de 20 $g.l^{-1}$ de saccharose et de 20 $g.l^{-1}$ de charbon actif (Merck 2186).

TABLEAU

Inventaire des microgreffes vivantes 2 mois après le microgreffage *in vitro* pour chacun des 4 génotypes étudiés.

Scion survival rates 2 months after the in vitro *micrografting for each of the 4 genotypes tested*

N° d'identification et âge du génotype greffé	Effectifs de microgreffes réalisées	Proportions de greffons vivants après 2 mois
n° 297 - 11 ans.	72	47/72 soit 65 %
n° 3110- 80 ans.	31	25/31 soit 80 %
n° 4301- 80 ans.	21	12/21 soit 57 %
n° 107 -100 ans.	62	43/62 soit 69 %

L'ensemble a été préalablement autoclavé 20 mn à 120°C. Les cultures sont ensuite replacées en conditions d'environnement initiales.

RÉSULTATS. — La technique de microgreffage a été appliquée à quatre génotypes d'âges différents. Les effectifs respectifs sont indiqués à titre indicatif dans le tableau. L'inventaire effectué 2 mois plus tard dénombre globalement 127 greffons vivants organogènes sur 186 microgreffes, soit 68 % de réussite.

Dans les situations favorables, l'union s'effectue précocement par prolifération des tissus corticaux entaillés du porte-greffe, prélude à la reprise d'activité organogène du méristème greffé sous forme d'une phyllogenèse active (*fig.* 2 A, B et C). Bien que les effectifs expérimentaux observés ne permettent pas de conclure quant à l'influence de l'âge sur la reprise du greffon, il semble que les méristèmes greffés provenant des génotypes les plus âgés évoluent préférentiellement vers la production de bourgeons écailleux. Ceux-ci, éventuellement entourés d'un ou deux brachyblastes, sont susceptibles de demeurer en repos végétatif apparent plusieurs semaines, voire plusieurs mois. Les greffons prélevés sur du matériel de 11 ans réagissent plus rapidement, parfois au bout de 1 à 2 semaines, en développant une pousse feuillée garnie d'euphylles à morphologie juvénile (*fig.* 2 D). Au vu de nos premières observations, le greffon exprimerait des caractéristiques d'autant plus juvéniles que sa reprise à l'issue du microgreffage est précoce. Néanmoins, une très grande hétérogénéité de réponses subsiste au sein d'un même génotype.

La croissance du greffon est favorisée par un sevrage progressif afin d'éviter une trop forte concurrence avec l'appareil caulinaire du porte-greffe. Les pourcentages de reprise lors de l'acclimatation en serre varient entre 80 et 100 % en fonction de la saison et grâce à la qualité de l'appareil racinaire du porte-greffe développé *in vitro* en Sorbarod.

DISCUSSION ET CONCLUSION. — En 1986, nous proposions une nouvelle technique de microgreffage permettant de propager végétativement avec 35 % de succès des *Sequoiadendron giganteum* centenaires à partir de points végétatifs [10]. Les résultats exposés montrent que cette méthode est applicable au Pin maritime, en l'adaptant aux particularités spécifiques et en l'améliorant par la même occasion.

Ainsi, le support de culture Sorbarod, destiné à faciliter les manipulations de microgreffage sans léser l'appareil racinaire et tout en garantissant sa neutralité chimique vis-à-vis du milieu de culture *in vitro*, présente l'avantage d'être biodégradable en conditions horticoles et facilite de ce fait l'acclimatation.

Par ailleurs, la sécrétion d'exsudat au niveau des tissus entaillés permet de simplifier considérablement les manipulations par rapport à *Sequoiadendron giganteum* ([10], [11]), en gagnant en rapidité d'exécution et en augmentant simultanément les taux de réussite. En fait, l'union entre le porte-greffe et le greffon, réduit au méristème caulinaire apical sans embase, s'effectue précocement de façon très naturelle en l'absence de toute substance de croissance exogène, antioxydant et autres artifices avantageusement employés dans d'autres cas ([9], [20], [21]).

Ces divers arguments nous incitent à développer cette technique convoitée [20] qui permet de cloner des Pins maritimes sélectionnés âgés à partir de méristèmes dans des proportions bien supérieures aux résultats obtenus sur milieux de culture artificiels [8]. De plus, cette méthode paraît transposable dans son principe aux autres espèces du genre. Certains aspects méritent néanmoins d'être analysés de façon plus approfondie, tels que l'influence de l'âge du pied-mère originel sur le devenir des méristèmes greffés et leur aptitude à recouvrer certains caractères juvéniles. Par ailleurs, l'hétérogénéité de réponses constatée à l'intérieur d'un même génotype devrait pouvoir être minimisée en accordant plus d'attention au stade physiologique du méristème au moment du prélèvement au sein du bourgeon écailleux [21]. Ces principaux thèmes d'investigation sont actuellement étudiés dans notre laboratoire.

Note remise le 18 septembre 1989, acceptée après révision le 9 novembre 1989.

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Rejuvenation of a 100-year-old *Sequoiadendron giganteum* through in vitro meristem culture. I. Organogenic and morphological arguments

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Monteuuis, O. 1991. Rejuvenation of a 100-year-old *Sequoiadendron giganteum* through in vitro meristem culture. I. Organogenic and morphological arguments. – Physiol. Plant. 81: 111–115.

True-to-type cloning of mature trees, especially when they do not sprout from their base, remains problematic. Special attention is focused on the shoot apical meristem, since it is an obvious choice for vegetative propagation. In *Sequoiadendron giganteum* a meristem removed during budbreak from a 100-year-old tree regenerated a truly rejuvenated line that exhibited the same juvenile characters as the juvenile clone used as control, especially in regard to morphological traits and organogenic capacity, and as manifested by the ability to produce adventitious roots in vitro. This rejuvenation has been maintained for 3 years in both in vitro and ex vitro conditions. This result is discussed in terms of inhibitory correlative systems acting within the donor tree in situ, especially as concerns miniaturization of the explant.

Key words – Juvenile, mature, meristem, micropropagation, morphological traits, organogenic capacities, rejuvenation, *Sequoiadendron giganteum*.

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Introduction

True-to-type cloning of selected mature trees is recognized as a powerful tool in forest tree improvement (Zobel and Talbert 1984). In practice, major problems exist because the ability for vegetative reproduction declines when the tree reaches a stage of development that allows a reliable assessment of its real value (Bonga 1982). The phenomenon has been studied in the giant sequoia, *Sequoiadendron giganteum*, an interesting forest species as regards its crop potential (Franclet et al. 1980).

In order to stimulate the capacity for true-to-type cloning of superior trees, various rejuvenation techniques have been tested. Manipulations such as grafting onto young seedlings, serial propagation by cuttings and microcuttings cultured in vitro have proved unsuccessful in achieving satisfactory rejuvenation (O. Monteuuis 1988, Ph. D. Thesis, Univ. B. Pascal, Clermont-Ferrand, France). This prompted us to select juvenile tissues remaining within the mature ortet and to propagate them preferentially. As is the case for most conifers, but in contrast to *Sequoia sempervirens*, *Sequoiadendron giganteum* does not sprout from its base. Therefore, special attention has been devoted to the shoot apical meristem, which is presumed to play a key role in physiological phase changes and rejuvenation (Schaffalitzky de Muckadell 1959, Bonga 1982,1987), on the presumption that it contains juvenile cell territories (Margara 1982).

For these reasons, meristem culture in vitro was attempted on a selected mature giant sequoia.

Materials and methods

Plant material

The mature material consisted of a clone obtained by grafting of scions taken from the crown of a 100-year-old *Sequoiadendron giganteum* Buchholz. This clone

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exhibited all the characteristics connected with maturity, including plagiotropic growth, small, hard and thick foliage, and an incapacity for adventitious rooting. The juvenile clone used as control derived from rooted cuttings from a two-year-old *Sequoiadendron giganteum* seedling. Previous papers (Monteuuis 1985, Monteuuis and Bon 1986) have given more detailed information about these two characterized experimental materials, which grow outdoors at the University of Clermont Ferrand, France.

About 300 terminal stem segments were removed during the rest period and at the time of budbreak from these cloned stock plants (see Monteuuis 1987a), and surface sterilized by soaking briefly in 70% ethanol. Shoot apical meristems were excised aseptically under a cold light source and immediately placed on the initiation medium. The term meristem refers to the apical dome and the youngest leaf primordia; any surrounding young leaflets were removed. Its size did not exceed 200–250 μm overall.

Culture conditions in vitro

The composition of the different culture media used chronogically to obtain rooted cuttings from excised apical meristem in vitro was described by Monteuuis and Bon (1986) and Monteuuis (1987a). At the end of the 3 weeks required for initiation on a basal nutrient medium (LP/2), organogenic meristems were transferred monthly to a half-strength medium (LP/4), until the explants reached about 1 to 2 mm. At this stage, they were placed on the elongation medium (EM). Multiplication by axillary budding occurred on the same EM medium after decapitation of the elongating shoots while transferring the upper parts to a fresh medium every 8 weeks. Microcuttings of 10 to 15 mm were used for rooting according to the following procedure: 3 weeks on root induction medium (MRI) containing 1 mg l^{-1} naphtalene acetic acid (NAA), then transfer to the same medium without NAA (MRE) (Monteuuis and Bon 1986). All these media, except MRI, lacked growth regulators. The cultures were maintained at 20 ± 2°C under a 16 h photoperiod with a photon flux density of 60–70 μmol m^{-2} s^{-1} provided by "Mazda-Fluor Lumière du jour" fluorescent lamps.

Acclimation

Microcuttings rooted in vitro were transferred to a peat/perlite (50/50, v/v) potting substrate and maintained at 23 ± 3°C under Sylvania Gro-Lux lighting with a photon flux density of 60–70 μmol m^{-2} s^{-1} under a 16 h photoperiod and with 80–85% relative humidity. About 6 weeks later, the plantlets were potted in a bark/peat substrate (70/30, v/v) and cultured first in a greenhouse, then outdoors.

Results

Capacity for organogenesis

The capacity of excised meristems to develop new shoots in vitro was greatly influenced by the age of the plant material and time of explant removal, with best results corresponding to the juvenile clone and to budbreak (Monteuuis 1987a). Elongation ability of the mature material was particularly weak, and the few shoots that were obtained with difficulty after several months showed mature characteristics. Nevertheless, one meristem from the mature clone removed during budbreak responded as though juvenile, exhibiting the same organogenic potential to regenerate a vigourous shoot. Subsequent observations denoted similar ability for axillary budding, corresponding to multiplication rates of 2.5 to 3 for every 8-week subculture cycle on elongation medium. Hence, 20 months after its introduction on initiation medium, this high-potential meristem gave rise to 280 reactive microcuttings. Some of these microcuttings rooted spontaneously on elongation medium with the same frequency, estimated at 6 to 7% (data not shown), as for the juvenile clone. This ability to root must be objectively considered as a juvenile character (Fouret et al. 1986). Growth activity was the same as for the juvenile material with fluctuations during time course (O. Monteuuis 1988, Ph. D. Thesis, Univ. B. Pascal, Clermont-Ferrand, France). Ability for adventitious rooting was examined after induction on rooting media. Table 1 shows no significant differences in rooting rates between the juvenile clone and the rejuvenated line (on average 46.1% and 44.3%, respectively), while under the same conditions, the original mature form proved to be totally recalcitrant (Monteuuis and Bon 1986, Monteuuis et al. 1987). Organogenic similarities between the rejuvenated material and the juvenile control persisted ex vitro, since 90–95% of the plantlets rooted in vitro were successfully acclimated, subsequently developing vigourous orthotropic leader shoots. In addition, it should be noted that in a preliminary assessment of growth, the orthotropic rejuvenated rooted cuttings were greatly advantaged as compared to the original

Tab. 1. Rooting data for the rejuvenated line and for the juvenile clone used as control, which were systematically paired during all culture procedures. The original mature clone proved to be totally recalcitrant for adventitious rooting under the same experimental conditions.

Dates of rooting experiments	Rejuvenated line	Juvenile clone (control)
1988-06-02	11/18	10/18
1988-08-01	8/24	11/24
1988-10-27	7/22	12/23
1989-01-16	13/24	8/24
Average mean	39/88	41/89
% ± SD	44 ± 5	46 ± 5

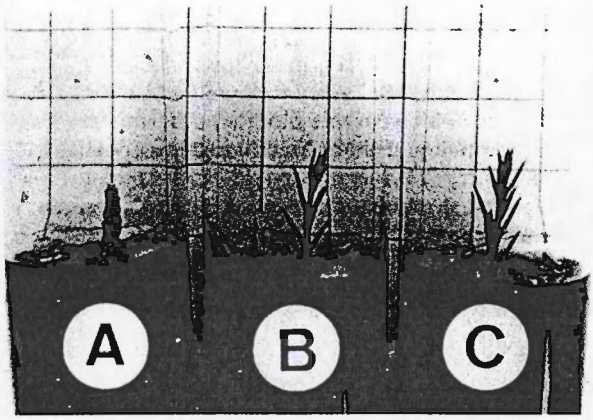

Fig. 1. Morphological differences on elongation medium between microcuttings of: 100-year-old genotype (A), the same genotype as in A rejuvenated throughout meristem culture (B), and the juvenile clone used as control (C). The sides of the background squares are 1 cm long.

mature form of the same genotype obtained by grafts and which was growing plagiotropically.

Morphological considerations

The reactive meristem of the mature clone from which the rejuvenated line originated exhibited very early morphological traits characteristic of the juvenile status (Monteuuis 1985). The first leaves formed were long and thin, and comparable to those of the juvenile clone, whereas by contrast the mature shoots possessed a foliage of small, hard and thick leaves (Fig. 1). The foliar similarities with the juvenile control persisted homogeneously in the culture in vitro. The newly formed root system appeared to be of equivalent quality, with many long and thin adventitious roots produced by both the juvenile and the rejuvenated clone. Morphological analogies between these two materials persisted after acclimation under outdoor conditions, the rooted cuttings of the juvenile clone and of the rejuvenated line exhibiting orthotropic growth and characteristic juvenile foliage. This was in contrast to the original grafted material that belonged to the same genotype, remaining plagiotropic with thick and hard leaves (Fig. 2). Particular attention was devoted to leaf length, which was attested to be a reliable marker of the degree of maturation (Monteuuis 1985) and was found to be greatly influenced ($P < 0.0001$; Fischer's test) by the origin of the investigated plant material. In fact, as shown in Tab. 2, leaves of the rejuvenated line, similar to those of the juvenile clone, were much more developed than those belonging to the original mature form.

Tab. 2. Mean leaf length (n = 50) for the 100-year-old genotype, the rejuvenated form, and the juvenile clone (control). Data are expressed ± confidence interval for $P = 0.05$. Means followed by the same letter do not differ significantly at the 5% level.

Origin of material	Leaf length (mm)
100-year-old genotype	1.40 + 0.18a
Rejuvenated form	5.58 + 0.42b
Juvenile clone	5.60 + 0.37b

It should be emphasized that the striking analogies observed between the juvenile control and the rejuvenated line deriving from the 100-year-old genotype, have since been maintained in either in vitro or ex vitro conditions, corresponding to 4 and 3 years, respectively.

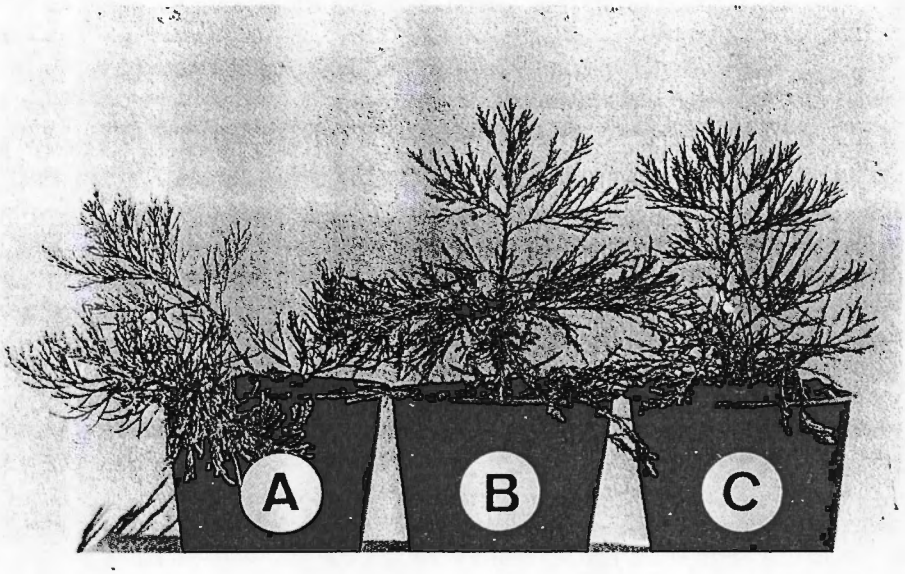

Fig. 2. Morphological features of the three types of experimental materials in in vivo conditions. The grafted 100-year-old genotype (A) exhibits characteristic plagiotropic growth and has small, hard and thick leaves by contrast with cuttings of the rejuvenated line (B) and the juvenile clone (C). The plants are potted in 5 l containers.

Discussion

As far as we are aware, this is the first time that rejuvenation has been demonstrated so clearly for a 100-year-old coniferous species that normally does not sprout. In fact, the reversion towards the juvenile stage represented by the juvenile clone proved to be stable and was verified biochemically (Bon and Monteuuis 1990), allowing this phenomenon to be considered a true rejuvenation. It differs from the ephemeral and partial 'retours en arrière' (Nozeran 1978) that result from subculture procedures with experimental in vitro media (Fouret et al. 1986) or consequently to grafting onto young seedlings (O. Monteuuis 1988, Ph. D. Thesis, Univ. B. Pascal, Clermont-Ferrand, France), effects that have been observed to diminish with subsequent elongation of the shoot, especially after acclimation (Mullins et al. 1979, Y. Fouret 1987, Ph. D. Thesis, Univ. P. et M. Curie, Paris, France). In this context, two factors appear to have a major influence in rejuvenation: the size of the explant and the period of removal from the stock plant (O. Monteuuis 1988, Ph. D. Thesis, Univ. B. Pascal, Clermont-Ferrand, France).

Concomitant comparative investigations conducted simultaneously both on the juvenile and on the mature clone pointed out that the significant differences existing at the shoot tip level between the two materials tended to disappear at budbreak time (Monteuuis 1987b, Monteuuis and Gendraud 1987, Monteuuis 1989), and to reappear as the shoots began to elongate. These observations support the interpretation of the maturation process affecting the shoot apical meristem according to a reiterative pattern closely related to the dynamics of shoot expansion, as proposed first by Krenke (1940) and then by Franclet (1985). Referring to these authors, all the organogenic apical meristems at the origin of the morphogenic programme should be able to express reiterative space-time juvenile potentialities during a period that shortens with the increasing ontogenetic development of the tree, and exhibiting the maximum intensity at the time of budbreak. In terms of probability, these period and tissue restrictions for occurrence of juvenility could explain the relative failure of rejuvenation attempts from the meristem culture of mature trees (Bekkaoui et al. 1985, Monteuuis 1987a), even when taking account of limiting technical aspects. In fact, the rejuvenation obtained consisted in removing the meristem from the mature ortet at the physiological period which corresponded to the maximum level of juvenile potentialities (Bonga 1987). Thus, the miniaturization of the explants through meristem culture in vitro appears as an advantageous means to counteract the presumed negative effects of a physiologically inhibitory environment in situ, that could suppress the possibility for the apical meristem to express its inner juvenile potentialities at certain periods (Nozeran 1978, 1984). Capacities for vegetative propagation of the explants could be largely influenced by their physiological status, which is susceptible to fluctuation during the time course as illustrated by variation in rootability (Tab. 1), previously reported for juvenile material (Monteuuis and Bon 1986, Monteuuis et al. 1987). This occurrence of a favourable physiological state to ensure true rejuvenation from excised meristems convinces us, in agreement with Nozeran (1978,1984) and Bonga (1987), that the miniaturization of the explant seems to be a necessary but not sufficient condition to achieve rejuvenation.

In conclusion, the aim of the present paper was to demonstrate that rejuvenation from a 100-year-old *Sequoiadendron giganteum* was achieved (see also the relevant biochemical arguments by Bon and Monteuuis 1990). Additional investigations are underway to define more precisely the advisable time of removal of the meristems from the donor trees, so that the phenomenon may be reproduced with enhanced success rates for other species. The organogenic capacity of the rejuvenated line obtained from the single meristem is sufficiently high to ensure large-scale true-to-type cloning of the mature selected ortet.

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Rejuvenation of a 100-year-old *Sequoiadendron giganteum* through in vitro meristem culture. II. Biochemical arguments

Marie-Claude Bon and Olivier Monteuuis

Bon, M. C. and Monteuuis, O. 1991. Rejuvenation of a 100-year-old *Sequoiadendron giganteum* through in vitro meristem culture. II. Biochemical arguments. – Physiol. Plant. 81: 116–120.

The phenomena of phase change and rejuvenation are characterized mainly by morphological and physiological criteria. Thus far, biochemical assessments have been relatively limited. In *Sequoiadendron giganteum*, techniques of sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and miniaturized two-dimensional gel electrophoresis were applied to a meristem-derived line from a 100-year-old tree to attest the basic origin of the resulting rejuvenation observed from morphological and organogenic standpoints in vitro as well as after acclimation in outdoor conditions. The membrane-associated protein J16, which characterizes the juvenile status was detected in both the juvenile control and the rejuvenated line, while in the original mature form it was totally lacking. In addition, two-dimensional electrophoretic analysis of protein patterns of single meristems belonging to the mature and the rejuvenated form suggested that rejuvenation might involve a drastic modification of the protein content within the meristem itself.

Key words – Biochemical markers, J16, membrane fraction, meristem, two-dimensional gel electrophoresis, polypeptides, rejuvenation, *Sequoiadendron giganteum*.

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Introduction

The phenomenon of phase change and the hypothetic rejuvenation of woody plants have been extensively described, in respect mainly to morphological characteristics and physiological manifestation (Bonga 1982, Hackett 1985). Much effort has been devoted recently to analyse the basic events underlying these features. The involvement in the maturation process of endogenous plant growth regulators (Paton et al. 1970, Rogler and Hackett 1975, Y. Fouret 1987, Ph. D. Thesis, Univ. P. et M. Curie, Paris, France), phenolic compounds (Jay-Allemand 1988), or the pentose phosphate pathway (Drouet et al. 1989) have been pointed out. In *Sequoiadendron giganteum*, Monteuuis and Gendraud (1987) and Bon (1988a), respectively, reported on different ATP/NTP ratios in winter, and GTP and GDP contents in spring in juvenile and mature material.

It has also been noticed that mesophyll cell walls of mature material contained more polysaccharides than those of young material (Monteuuis and Genestier 1989). In view of previous research (Bon 1988b), it appeared that a membrane-bound polypeptide of 16 kDa ('J16') could be identified as a reliable marker of juvenility for *Sequoiadendron giganteum*.

Concurrently, use of meristem culture in vitro for true-to-type cloning of a mature 100-year-old *Sequoiadendron giganteum* gave rise to a very responsive plantlet line that exhibited striking morphological and organogenic analogies with the juvenile plants used as control (Monteuuis 1990). These features prompted us to investigate this material biochemically in order to determine the basic origin of the hypothesized rejuvenation.

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Materials and methods

Plant material

The experimental material of *Sequoiadendron giganteum* Buchholz derived from Monteuuis (1990). It consisted of a juvenile clone as control, a 100-year-old mature clone, and its rejuvenated line. All the plants, except the mature clone obtained by grafts, originated from meristem culture under the same in vitro conditions.

Biochemical methods

Samples were collected during the rest period from the 3 types of plant material that had been cultured under similar horticultural conditions since they were acclimated 3 years earlier (Monteuuis 1990). Techniques of SDS-PAGE and miniaturized 2D-PAGE involved, respectively, 400 mg of apices and one single apical meristem for each type of plant material. All experiments were repeated at least 3 times at weekly intervals.

Protein extraction and SDS-PAGE

Shoot apices with surrounding young chlorophyllous leaves were removed and ground in a mortar with a pestle. A crude membrane fraction was obtained as previously described by Bon (1988b). Protein was assayed according to Bradford (1976) using bovine serum albumine as standard in order to load the same protein concentration in each gel lane. Proteins of the fraction corresponding to the membrane-enriched pellet were separated by SDS-PAGE (12% running gel, 4% stacking gel) as described by Laemmli (1970). The electrophoresis was carried out at 120 V for 12 h and the gel stained with silver according to the method applied by Bon (1988b).

Micro two-dimensional electrophoresis

For each two-dimensional electrophoresis, a single meristem of 200–300 µm, consisting of the apical dome and the two youngest primordia, was removed and the proteins extracted according to Bon (1989a). The procedure for two-dimensional electrophoresis was as described by Bon (1989a). Iso-electric focusing in the first dimension was performed with gels composed of 3.78% (w/v) acrylamide, 0.22% (w/v) N,N'-methylenebisacrylamide, 2% Triton X-100, 9.2 *M* urea and 4% (v/v) carrier ampholytes (1/4 Pharmalytes pH 5.0 to 6.0 and 3/4 pH 5.0 to 8.0) and carried out for 5000 Vh. SDS-PAGE in the second dimension was obtained with the system of Laemmli (1970), and the resultant gels silver-stained.

Results

Membrane protein patterns

In order to estimate the biochemical changes underlying the rejuvenation process, we partially purified the membrane fraction. Since previous data indicated that the J16 protein belonged to this fraction, the same extraction technique was used as previously reported (Bon 1988b). Polypeptides from the membrane-associated proteins were also separated by SDS-PAGE. A protein band corresponding to the J16 protein was observed in the juvenile clone (control) and in the rejuvenated line, whereas it was not detected in the original mature form (Fig. 1).

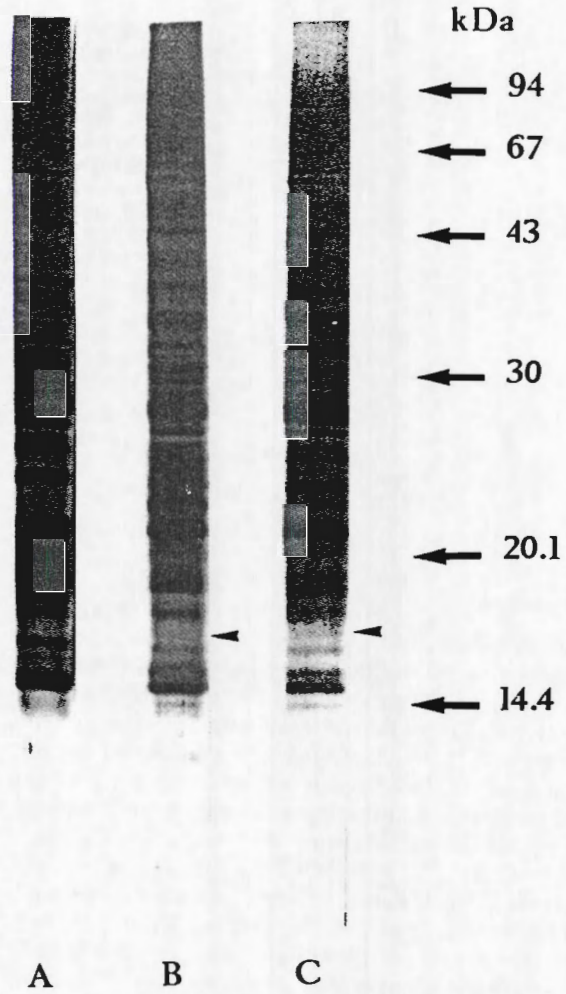

Fig. 1. Silver-stained SDS-PAGE profiles of membrane-associated polypeptides of: 100–year-old genotype (A), the same genotype rejuvenated through meristem culture (B), and the juvenile clone used as control (C). Equal amounts of proteins were loaded in each gel lane. The 'J16' protein is indicated by arrowheads.

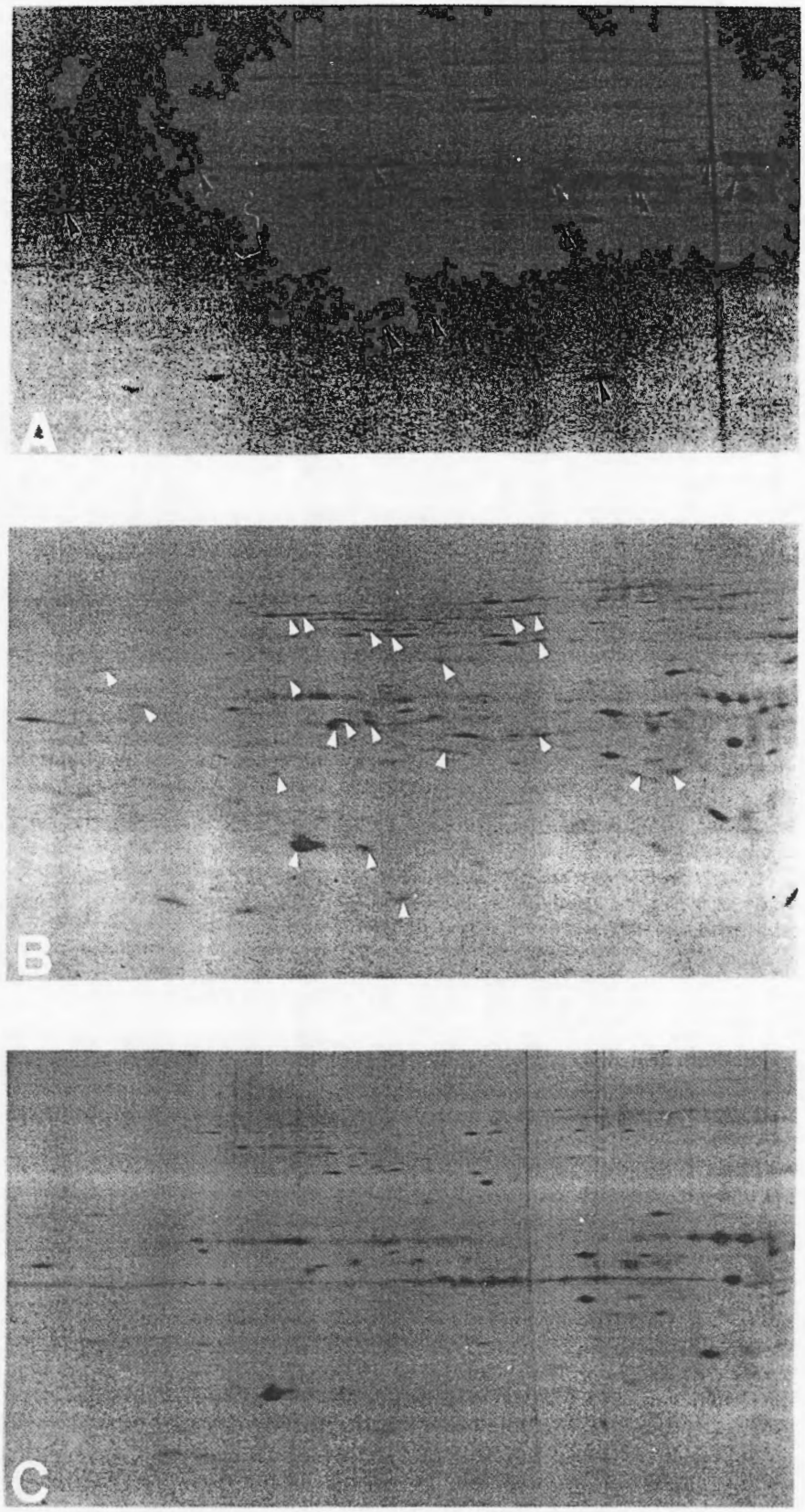

Fig. 2. 2-D protein patterns obtained from a meristem belonging to the 100-year-old genotype (A), its rejuvenated meristem derived line (B) and the juvenile clone used as control (C). Black arrowheads indicate spots present or more intense in (A) but absent or less intense in (B). White arrowheads indicate spots absent or less intense in (A) as compared to (B).

Comparison of two-dimensional protein patterns in the apical meristem

In order to assess the hypothetical influence of the phase change within the inner apical meristem itself, two-dimensional protein patterns of meristems belonging to the original mature form and to the rejuvenated line were compared. For each clone, the polypeptide variation among the replicates was evaluated to 10% of the whole population. Only polypeptides common to the 3 replicates were taken into consideration to assess the clonal differences.

The rejuvenated mature clone displayed a few more spots than the original 100-year-old clone (Fig. 2A and B). Moreover, the profiles observed revealed that within a meristem of the rejuvenated line, 10 spots appeared and 12 increased in intensity while 8 spots disappeared and 7 decreased in intensity as compared to the same genotype in the original mature form. Most of the qualitative and quantitative differences (in terms of the 37 spots previously described) observed between the rejuvenated and the mature form were analogous between the control (juvenile) and the mature clone (Fig. 2B and C).

Discussion

It is an advantage if basic biochemical investigations of phase change phenomena are carried out on the same genotype. In the case of *Sequoiadendron giganteum*, a tree that does not produce 'rejuvenated' stump sprouts (Bonga 1982, Jay-Allemand 1988), the availability of a rejuvenated meristem derived line from a 100-year-old tree makes this possible.

The membrane-associated 16 kDa polypeptide, termed J16 (Bon 1988b,1989b), was present in the juvenile control and the rejuvenated form, but not in the original mature clone.

In ivy, Fukasawa (1966) has highlighted different protein bands in callus according to the age of the plant. However, only a few data were related to the biochemical aspect of the rejuvenation process (Y. Fouret 1987, Ph. D. Thesis, Univ. P. et M. Curie, Paris, France, Jay-Allemand 1988). As far as we know, this is the first report on the use of a protein to confirm the biochemical basis of the rejuvenation observed in a conifer, the organogenic and morphological aspects of which are described elsewhere (Monteuuis 1990). The presence of this particular protein appeared to be independent of the physiological state of the rejuvenated material. So far, neither the nature and function of this membrane-associated polypeptide nor the relevant factors involved in induction or enhancement of the rejuvenation associated gene expression are known.

In this connection, sequences encoding the chlorophyll *a/b* binding protein of photosystem II are differentially expressed in juvenile and mature trees of Larix (Hutchison et al. 1989). However, no information exists concerning the incidence of the rejuvenation on the expression of these sequences.

As observed, the reversion from the mature to the juvenile phase in *Sequoiadendron giganteum* involved significant differences between the two-dimensional protein patterns of the two materials at the level of the apical meristem. Earlier, we (Bon and Monteuuis 1987) showed that the foiiar dimorphism considered as a morphological marker of phase change could be associated with specific polypeptides. The present investigation has confirmed that such polypeptides, connected with morphological and organogenic rejuvenation, are found in the juvenile control. The identification of the patterns of proteins of the rejuvenated line with those corresponding to the juvenile control suggested that expression of genes for these polypeptides might be involved in the rejuvenation phenomenon.

As hypothesized by Monteuuis (1989,1990), miniaturization of the explant and the appropriate physiological stage of removal seem to be two determinant parameters in achieving rejuvenation. Although rejuvenation may be associated with a synthesis of some particular polypeptides, it is difficult to attribute specific proteins to each of these two parameters.

To conclude, in addition to organogenic and morphological evidence, it appears that the rejuvenation resulting from the meristem culture of a 100-year-old giant sequoia involves biochemical reversions that have an undeniable analogy with that in juvenile material. Such findings seem to be obvious arguments to support the basic origin of the phenomenon.

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NOTES

Morphological features as indicators of maturity in acclimatized *Pinus pinaster* from different *in vitro* origins

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MONTEUUIS, O., and DUMAS, E. 1992. Morphological features as indicators of maturity in acclimatized *Pinus pinaster* from different *in vitro* origins. Can. J. For. Res. 22: 1417–1421.

Acclimatized *Pinus pinaster* Ait. plants originating from five different *in vitro* culture origins were compared morphologically. After one growing season in the greenhouse, the plants displayed the following order of maturity (from juvenile to mature): (*i*) young seedlings; (*ii*) plants produced by adventitious budding from cotyledons; (*iii*) plants derived from micrografted meristems of 2-year-old seedlings; (*iv*) plants derived from micrografted meristems of a 13-year-old mature clone; and (*v*) plants derived from microcuttings of axillary shoots of the same 13-year-old mature clone. Plants derived from micrografts exhibited substantial variability with regard to morphological characteristics. This variability included some striking demonstrations of morphological rejuvenation among the mature clone micrografts which, on average, showed more juvenile traits than the microcuttings of the same clone.

MONTEUUIS, O., et DUMAS, E. 1992. Morphological features as indicators of maturity in acclimatized *Pinus pinaster* from different *in vitro* origins. Can. J. For. Res. 22 : 1417–1421.

Cinq échantillons de vitroplants de *Pinus pinaster* Ait. acclimatés ont été comparés, en fonction de leurs origines respectives, sur base de critères morphologiques. À l'issue d'une saison de végétation en serre, le classement selon un degré de maturité croissant s'établit comme suit : (*i*) jeunes semis; (*ii*) tigelles issues de bourgeonnement adventif sur cotylédons; (*iii*) microgreffes de méristèmes de semis de 2 ans; (*iv*) microgreffes de méristèmes d'un clone mature âgé de 13 ans; (*v*) microboutures issues du bourgeonnement axillaire *in vitro* du même clone mature. Les microgreffes présentent dans l'ensemble une grande variabilité susceptible d'handicaper, pour certains critères d'analyse, la discrimination en fonction de l'âge du matériel d'origine. Cette hétérogénéité inclue des cas de rajeunissements morphologiques tout-à-fait spectaculaires au sein des microgreffes du clone mature qui, dans l'ensemble, présentent plus de caractères juvéniles que les microboutures du même clone à physionomie mature caractérisée.

Introduction

Pinus pinaster Ait. must be considered as a major forest pecies, with increasing crop potential in many countries. In rance, it is the most important conifer from the standpoint f afforestation. As with other forest species, vegetative propgation may be of value as a means of improving the quality f plantations (Chaperon 1980). But in practice, conventional egetative propagation methods often fail in achieving true o type cloning of mature selected individuals (Rauter 1983). arious *in vitro* propagation methods have been developed in ur laboratory, including protocols for adventitious budding, neristem micrografting, and multiplication by axillary budling. In this paper, we assess the quality of plants from five *n vitro* origins after acclimatization to *ex vitro* conditions. pecial attention is given to the degree of maturity, which is n important factor in true to type cloning (Franclet 1983). or this evaluation, we used morphological and morphoenetic traits that are related to ontogenetical development Debazac 1963) and reflect aging of shoot apical meristems Schaffalitzky de Muckadell 1959; Hackett 1985; Monteuuis 989). In *P. pinaster*, as well as other pine species, juvenile lants are characterized by a continuous growth activity (free rowth), because the elongation of the shoot and the initiation f new vegetative structures occur simultaneously (Gaussen 950; Thompson 1976). As seedlings age, there is more of tendency toward successive flushes (Kozlowski 1971) or polycyclism (Debazac 1963), indicating the end of the strictly juvenile period (Greenwood 1987). The frequency of flushes diminishes as trees become more mature (Alazard 1980; Greenwood 1984; Foster *et al.* 1987), and the total number of lateral branches also decreases (Bolstad and Libby 1982; Greenwood 1984; Struve and McKeand 1990). The morphology of terminal buds also reflects the maturation phenomenon. The open, leafy buds in younger seedlings gradually give way to scaly buds between flushes of shoot elongation as trees age (Fielding 1970; Kozlowski 1971; Sweet 1973).

Needle characteristics are also closely related to maturation in *Pinus*. Juvenile and chlorophyllous primary needles appear during the first stages of development in young seedlings and become thicker, shorter, and less abundant, ultimately exhibiting scale or bractlike morphology as trees mature (Gaussen 1950; de Ferré 1952; Kozlowski 1971). At this time, needle fascicles are produced, which become the main photosynthetic organs of the tree (Gaussen 1950; Kozlowski 1971; Thompson 1976). Additionally, primary leaf length has been considered an indicator of juvenility (Burdon and Bannister 1985; Simons and Skidmore 1989) and also of capacity for vegetative propagation (Haines *et al.* 1989*a*, 1989*b*).

Materials and methods

Origin of plant material

Five types of *P. pinaster* plant material originating from the same provenance but derived from different explant ages and *in vitro* methods were compared for their morphological characteristics: (*i*) 4-month-old seedlings grown *in vitro* using the same conditions as the rootstocks

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Discussion

Although micropropagation of conifers has been rather widely studied, research on the further development of micropropagated plantlets after transfer to *ex vitro* conditions has been limited largely to *Pinus radiata* D. Don (Fielding 1970) and *Pinus taeda* L. (McKeand 1985; Frampton and Isik 1987). We chose to carry out such investigations soon after acclimatization because changes that affect further development of *in vitro* issued plants placed in *ex vitro* conditions can occur rapidly after transfer (McKeand 1985; Fouret 1987). In addition, for operational purposes, individuals for stock plants should be selected as early as possible.

We used young seedlings as a juvenile reference, as has been described by several authors (Gaussen 1950; de Ferré 1952; Kozlowski 1971; Thompson 1976). The seedlings were grown *in vitro* to minimize differences with the other plant types.

Considering all the various morphological features analysed with their significance in terms of indicators of maturity, the classification of the material from juvenile to mature was (*i*) seedlings (juvenile controls); (*ii*) plants derived from adventitious buds; (*iii*) 2-year-old seedling micrografts; (*iv*) 13-year-old clone micrografts; (*v*) 13-year-old clone microcuttings. This evaluation could be improved by weighing the different traits according to their importance to discriminate between the juvenile and mature phases.

The fact that plants derived from adventitious buds appeared more mature than seedlings agrees with previous reports (McKeand 1985; Frampton and Isik 1987). This result could be due to nonoptimal *in vitro* culture conditions (Wisnienski *et al.* 1986; Frampton and Isik 1987), which has been postulated to induce features indicative of early maturation, for instance the plagiotropic habit in *Pseudotsuga menziesii* (Mirb.) Franco (Goldfarb and Zaerr 1989).

Comparison of the materials derived from micrografts with those derived from other micropropagation techniques is particularly valuable because this is the first time to our knowledge that such acclimatized micrografted plants have been available in sufficient numbers to allow for reliable evaluations. On average, the micrografts were assessed to be more mature than both the seedlings and the plants obtained adventitiously from cotyledons. This may be because of the ages of the donor plants from which the meristems (scions) were removed, which were 2 and 13 years old. However, the micrografts appeared more juvenile than the microcuttings from the same clone by many morphological criteria. Moreover, there were few significant morphological distinctions between micrografts of meristems taken from 2- and 13-year-old trees. The differences that did occur between these two origins, for example bud type and number of lateral shoots, may be due in part to different original physiological stages of excised meristems at the time of removal. This factor was found to be critical, especially for mature meristems (Monteuuis 1989). The location of excised meristems on the donor tree may also influence meristem behavior (Hackett 1985). These factors, the variable quality of the graft connection, and the genetic diversity of the rootstocks may have contributed to the within-class variability observed in both types of micrografts, including some morphological rejuvenation occurrences for the mature clone micrografts. Such hypotheses deserve further study in future research on micrografting and other micropropagation techniques for conifers.

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Effect of Technique and Darkness on the Success of Meristem Micrografting of Picea abies

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Abstract

The possibility of micrografting *in vitro* shoot meristems of *Picea abies* were investigated on a 18 year-old Norway spruce clone. The rates of success were shown to be greatly influenced by the grafting technique used and by light, with a positive effect of a 2 to 3 week darkness period applied to the stocks just after they had been grafted. Average scores of more than 50% of success were obtained. However, substantial variability in terms of shoot expansion among the grafted plants existed *in vitro*, as well as after transfer to *ex-vitro* conditions.

Key words: micrografting, *Picea abies*, shoot meristem, technique, tissue culture, vegetative propagation.

FDC: 165.442; 174.7 Picea abies.

Introduction

There is much interest in favor of micrografting as broadly reviewed by Burger (1984) and Jonard (1986). With special regard to coniferous species, most of the work carried out in this field to date mentions the use of vegetative buds or shoot tips as scions (Misson and Giot-Wirgot, 1985; Tranvan and David, 1985; Ewald et al., 1991; Tranvan et al., 1991; Huang et al., 1992; Pullman and Timmis, 1992), but micrografting of shoot meristems has been restricted thus far to only a limited number of species (Monteuuis, 1986; Dumas et al., 1989; Goldfarb et al., 1993), despite the obvious benefits of miniaturizing the size of the grafted scion to the meristem. Meristem micrografting combines the advantages of grafting (Champagnat, 1980), with those of meristem culture, still problematic in practice for mature conifers (Pulmann and Timmis, 1992) to which it can constitute a helpful substitute. The possibility of introducing into tissue culture conditions contamination-free explants through grafted meristems derived from mature genotypes, while stimulating the potential for cloning of such introduced selected plant material at the same time (Franclet, 1983; Tranvan et al., 1991; Huang et al., 1992) must be considered a major argument for this technique. In addition, grafting meristematic tissues may help in reducing compatibility problems between the scion and the stock (Lachaud, 1975; Moore, 1984; Jonard, 1986).

The prospects of applying this attractive meristem micrografting technology to Norway spruce (*Picea abies* (L.) Karst.), a major forest species, were analysed and are reported in this paper.

Material and Method

Obtaining in vitro rootstocks

The *in vitro* seedlings used as rootstocks were obtained from *Picea abies* seeds that were surface-sterilized by immersion in 38 % hydrogen peroxide solution for 20 min, then rinsed 3 times in sterile distilled water before being individually inoculated into glass test tubes (25 mm x 200 mm) onto a 20 mm x 30 mm cellulosic "Sorbarod" plug (Baumgartner Papier SA, Lausanne. Switzerland). These Sorbarods had previously been saturated with 5 ml of liquid medium consisting of Margara (1977) macronutrients, Murashige and Skoog (1962) micronutrients diluted twice, 20 g/l sucrose and 10 g/l activated charcoal, before being autoclaved at 120 °C for 20 min.

The cultures were then maintained under a 16 h photoperiod with photon flux density for 110 μmol m^{-2} s^{-1} provided by "Sylvania Cool White" fluorescent lamps 36 W, and at 25/22 ± 2 °C, light/dark. Under these conditions, 50 % to 70 % of seeds that germinated developed within 2 to 3 months into young seedlings with fully expanded cotyledons and an elongating epicotyl that corresponded to the suitable stage to be grafted.

Scion origin

The apical meristems used as scions originated from vegetative buds produced by shoots of rooted cuttings of one AFOCEL superior clone of Norway spruce aged 18-years since seed germination. These 3-year to 4-year-old rooted cuttings were intensively maintained cultivated and hedged in large containers in a greenhouse with minimum temperature of 10 °C and permanent additional lighting provided by high-pressure sodium lamps.

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Several sample collections were carried out at different dates during the year.

Grafting technique

An soon as collected, the buds with a short portion of shoot underneath were dipped for a few seconds in a 70 % ethanol solution. The buds were then dissected aseptically and the apical meristems excised under a binocular microscope using a cold light source. The size of the apical meristems ranged from 100 μm to 250 μm in height and from 200 μm to 450 μm in width depending on growth phase of the shoot apex at the time of removal.

Two different micrografting techniques were compared (*Figure 1*): (a) the „side-grafting" originally developed on *Sequoiadendron giganteum* (Monteuuis, 1986) and consisting of inserting the excised meristem with a short wedge of underlying tissues into a 2 mm to 3 mm long vertical cut made on the elongating epicotyl of the in vitro seedling used as rootstock; (b) the "top-grafting" as initially described by Navarro et al. (1975) and consisting of placing the horizontal cut section of the excised meristem onto the top cut surface of the decapitated young epicotyl of the *in vitro* seedling rootstock. The overall size of the scion did not exceed 450 μm in width and 250 μm in height or 500 μm when removed with the basal wedge (side-grafting technique).

As soon as grafted for both procedures half of the rootstocks were placed under the same environmental conditions as formerly described, while the other half was kept for 2 to 3 weeks in darkness before being transferred to the standard conditions.

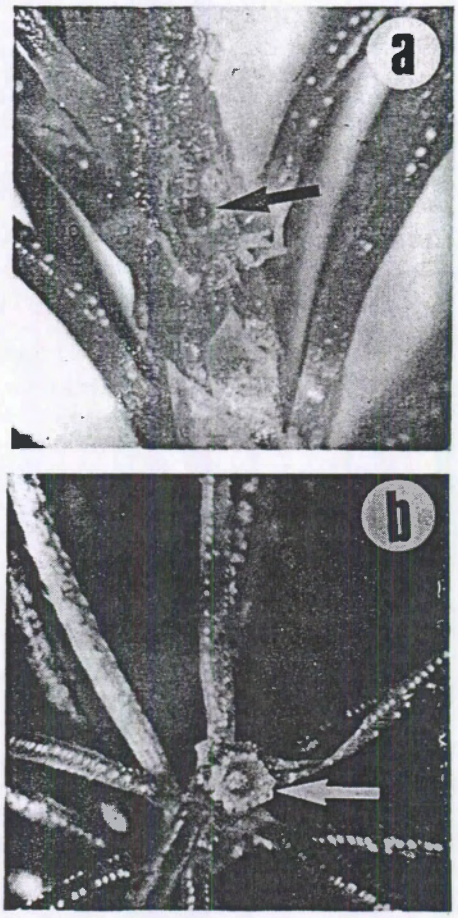

Figure 1. — Recently micrografted meristems of *Picea abies* (arrowheads) illustrating the "side-grafting" (a) and the "top-grafting" (b) techniques described in the text.

Figure 2. — Elongating scion deriving from a side-grafted meristem after the rootstock epicotyl had been cut back just above the graft point.

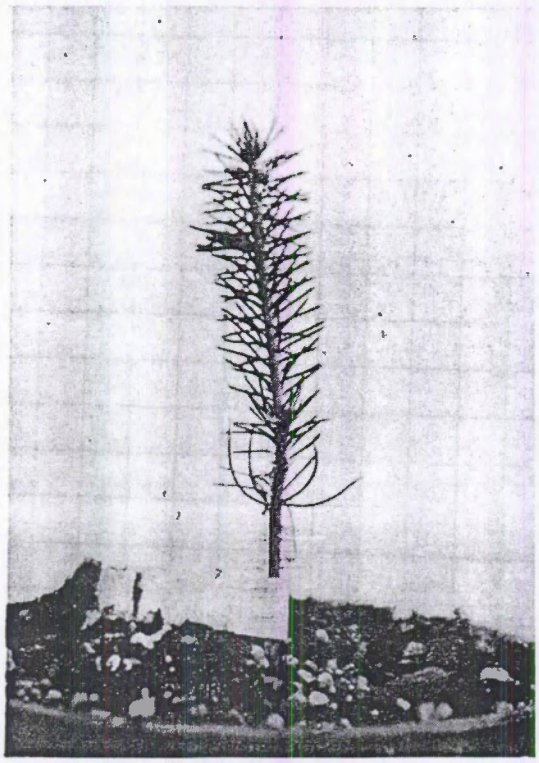

Figure 3. — Acclimated Picea abies micrograft. The sides of the background squares are 1 cm long.

The influence of darkness on grafting success was more precisely investigated by applying the same protocol to the side-grafted plants only.

Care of the grafted plants and acclimatization

Every 2 to 3 months, as required, 2 ml of the sterilized liquid medium was provided under aseptic conditions to the grafted rootstocks kept cultivated in their original test tube. In addition, soon after the side-grafted meri-

Table 1. — Micrografting success scores for side-grafting and top-grafting techniques applied to shoot meristems removed at different dates from an 18-year-old *Picea abies* genotype.

Dates of scion collection / grafting	Side-grafting	Top-grafting
13-6-1989	4/12	1/12
30-6-1989	6/12	0/10
25-7-1989	7/12	0/12
9-8-1989	8/12	2/12
29-12-1989	5/18	1/17
Average rate of success	30/66	4/63
% ± S.D.	45.5 ± 6.1	6.3 ± 3.0

SD: standard deviation

stem started new organogenesis demonstrating that connection with the stock had occurred, the stock epicotyl was cut off just above the graft point (*Figure 2*). Also any axillary shoot produced by the stock, whatever the type of grafting would be systematically removed to avoid competition.

Transfer of the grafted plants to the greenhouse was achieved preferably in spring, by carefully removing the sorbarod by hand before transplantation into a horticultural peat-perlite (50:50, v/v) substrate watered with a fungicide solution and covered with a thin plastic film to maintain sufficiently high relative humidity. This plastic cover was progressively removed after 2 weeks to 4 weeks to get the grafted plants acclimatized (*Figure 3*).

Results

The rate of success, in terms of meristems exhibiting organogenic capacity after they had been micrografted, was shown to be strongly influenced by the technique used, the side-grafting giving rise to 45.5 % of positive responses against 6.3 % for the top-grafting technique (*Table 1*, $P < 0.001$ as the result of the Chi-square test).

The beneficial influence of placing the grafted *in vitro* seedlings for 2 weeks to 3 weeks in darkness immediately after grafting resulted in average success rates of 52.4 % as compared to 32.6 % for the control in standard lighting conditions (*Table 2*, $P < 0.01$, Chi-square test).

However, it appears from *table 1* and *table 2* that the scores were susceptible to variation from one date of experiment to another.

Whatever the procedure used, the successful micrografts exhibited substantial variability in terms of further development of the scion, from a resting scaly bud to an actively expanding juvenile-like shoot. Such noticeable variability was observed even for meristems removed at the same date from the mother plant.

The grafted plants were acclimatized to *ex-vitro* conditions without any serious problem.

Discussion

The success of meristem micrografting of Norway spruce was shown to be largely dependent on the procedure used. The side-grafting technique appeared to be more efficient. It required high dexterity from the manipulator. Ability to draw the rootstock seedling out of the tube to facilitate manipulations without damaging its root system was a determining factor. This was possible thanks to the rod used as physical support. Furthermore, the better quality of the roots that developed in the "Sorbarod" made the transfer to the horticultural substrate easier. This allowed grafting onto juvenile seedlings, which can be considered more suitable than older material or unrooted microcuttings, giving higher rates of grafting success (Monteuuis and Dumas, unpublished results) and especially in view of the aim to recover juvenile potentialities from the grafted meristem assuming a transfer of graft-transmissible juvenility promoting substances from the young seedling used as stock (Champagnat, 1980; Dole and Wilkins, 1991).

In contrast to *Pinus pinaster* (Dumas et al., 1989), but in the same way as for *Sequoiadendron giganteum* (Monteuuis, 1986), the meristem had to be removed with a small wedge of underlying tissues to be inserted into the small cut previously made on the rootstock epicotyl to keep it in tight contact with the stock until the connection occured. The influence of these underlying tissues on the possibility of the grafted meristem to recover juvenile potentialities would require further analysis. But it seems logical to assume that the tinier the scion, the more damaging the excision and the more limited the endogenous resources needed until the connection with the stock has occurred thereby decreasing grafting success as demonstrated by Navarro et al. (1975) on *Citrus*, Huang and Millikan (1980)

Table 2. — Effect of 2 week post-grafting darkness on the success scores of meristem side-micrografts of an 18 year-old *Picea abies* genotype performed at different dates.

Dates of scion collection / grafting	Standard lighting conditions	Darkness
30-C-1989	2/6	4/6
25-7-1989	3/6	4/6
9-8-1989	4/6	4/6
24-8-1989	7/11	11/11
11-9-1989	7/9	4/7
27-10-1989	4/8	8/8
23-11-1989	2/11	4/10
11-12-1989	0/12	0/12
20-12-1989	0/11	0/9
29-12-1989	0/9	5/9
Average rate of success	29/89	41/84
% ± S.D.	32.6 ± 5.0	48.8 ± 6.4

SD: standard deviation

on *Malus*, and Monteuuis (1987) on *Sequoiadendron giganteum*. This could explain the failure of the meristems top-grafted onto the decapitated stocks, beside also the fact that the contact between the tissues of the 2 partners were not as tight as for the side-grafting method.

Endogenous growth regulators have been supposed to play a key role in grafting with special references to auxin (Shimomura and Fuzihara, 1977; Moore, 1984) which is known to be synthetized in shoot apices and degraded by light. This could constitute an hypothesis in favor of the beneficial effect of placing the grafted plants in darkness. in the same way as Tranvan and David (1985) and Tranvan et al. (1991), allowing for the time needed for the connection to occur. Light may also affect the excised meristem causing irreparable stress and photooxydation reactions.

The substantial intraclonal variability observed among the successful micrografted plants has been reported for other species when applying the same technology (Monteuuis, 1986; Dumas et al., 1989; Monteuuis and Dumas, 1992). It can be caused by several factors such as the variable quality of the graft connection, the genetic diversity of the seedlings used as rootstocks and the physiological status of the excised meristems at the time of its removal to be grafted. This latter parameter in particular seems worth requiring special attention with a view to remedying this undesirable variability. We deliberately decided to concentrate our efforts on 1 *Picea abies* clone with the purpose of minimizing this variability, at least from the standpoint o fthe scion source genotype. Another factor that obviously needs to also be considered is the influence of the *in situ* location of the scion within the donor plant.

Additional experiments have however established that this meristem micrografting technique can be successfully applied to other genotypes of Norway spruce and even likely to other related species.

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Rooting Acacia mangium Cuttings: Effects of Age, Within-Shoot Position and Auxin Treatment

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Summary

The possibilities of vegetatively propagating *Acacia mangium* through rooted cuttings were examined, focusing on 3 main factors, namely: (i) the age of the donor plant, comparing 6-month-old seedlings to sprouts from a mature stump, (ii) the original within-shoot position of the cutting before collection and (iii) the auxin treatment applied to the cuttings. These 3 factors were shown to influence greatly the capacity of the cuttings for rooting. The best scores in terms of rooting rates were obtained for the plant material collected from (i) the seedlings, (ii) the upper part of the shoot close to the terminal bud and (iii) when treated with auxin. The only interaction confirmed by statistical tests was between the age of the donor plant and the auxin treatment, with a greater auxin induced increment of the rooting rates for the cuttings from seedlings.

The results obtained tend to demonstrate that, although capable of improvement by optimizing the investigated factors, the potential of *Acacia mangium* to be propagated by rooted cuttings remains rather limited, especially when starting from mature material.

Key words: *Acacia mangium*, adventitious rooting, auxin, cuttings, maturation, vegetative propagation, within-shoot position.

FDC: 165.44; 161.4; 232.5; 176.1 *Acacia mangium*.

Introduction

Acacia mangium Willd., an arborescent species belonging to Leguminosae (Mimosoideae), is native to northern Australia (Queensland), western parts of Papua New Guinea and eastern provinces of Indonesia (Irian Jaya and Maluku) from sea level up to 720 m (Gunn and Midgley, 1991). It has been recognized as a valuable forest tree species since its initial introduction into Sabah (Malaysia) in 1966, where it has been demonstrated to thrive quite well, even on very acid and infertile soils whose fertility can be restored thanks to its natural nitrogen fixing ability. In such poor but rather frequent site conditions, it has been shown to outperform all the other fast growing forest tree species, especially in situations of proliferating weeds with which it competes successfully. The early vigorous growth of this short-lived pioneer enables it to reach commonly 20 m to 25 m in height within 10 years to 15 years in Sabah, with a wood production averaging 25 m^3/ha/year to 30 m^3/ha/year, despite the mediocre genetic value of the material planted so far (Sim, 1986). Owing to this remarkable potential, which accounts for the establishment of industrial plantations all over South-East Asia mainly for pulpwood production, it appears obvious as a matter of urgency to concentrate efforts on tree improvement of *A. mangium* with a view of improving the genetic quality of the planting stock. Progress in that field is just beginning and although simple breeding strategies based on sexual propagation seem objectively well adapted to the situation (Monteuuis and Nasi, 1992), the vegetative propagation option is also worth special attention. Theoretically, it can be used for mass "bulk" vegetative propagation of a restricted number of juvenile genotypes of presumably high genetic value — derived for example from controlled pollinations — (Wong and Haines, 1992), or for producing clones, either to establish clonal seed-orchards, or to develop clonal plantations, especially from genotypes selected from clonal tests of advanced generation families

(HAINES and GRIFFIN, 1992). However, although highly desirable in theory, the prospects of applying vegetative propagation to *A. mangium* are dependent on the ability of this species to be propagated by rooted cuttings.

So far, little information is available regarding this matter. However, it appears from previous studies (DARUS, 1991; HAINES et al., 1991) that the age of the plant material is likely to play a determining role, as with other arborescent species where the maturation process has been observed to act negatively on the potential for adventitious rooting (BONGA 1982; HACKETT, 1988). Another factor that should warrant special consideration according to WONG and HAINES (1991) is the initial position of the cutting within the shoot of the ortet — the donor plant it has been collected from — and distinguishing between the terminal-shoot cuttings and the single-node ones. The same authors reported also the beneficial effect of treating *A. mangium* cuttings with root-promoting exogenous substances, the so-called "auxins", specifically "Seradix 3", also used by DARUS (1991), to enhance the quantity and the rate of production of adventitious roots.

The influence as well as the possible interaction of these three factors on the rooting potential of *Acacia mangium* stem cuttings have been assessed.

Material and Methods

Softwood cuttings of *A. mangium* used for the rooting experiments were collected from 2 different donor plant types:

a. main stems of 6 month-old seedlings cultivated in the nursery;
b. 2 month-old sprouting shoots emerging from the stump of a 6 year-old ortet growing near the nursery.

The average size of the cuttings ranged between 6 cm to 8 cm in length and 3 mm to 5 mm in diameter. Except for the terminal-shoot with its apical bud, they consisted of one single node plus the full internode underneath. About half of the surface of the phyllodes — leaf-like petioles — was removed in order to lower evapotranspiration and to reduce water stress risks.

The original position of each cutting within the shoot was noted according to a basipetal numeration, with the terminal shoot cutting as number 1, the nodal segment cutting just below it as number 2, and so on down to number 8.

Half of the cuttings corresponding to each of the different origins used were treated with auxin by dipping their base into the Seradix 3 commercial powder preparation (0.8% of 3-indolebutyric acid in talc), whereas the remaining half was not treated to serve as control.

The next step consisted of inserting all the cuttings into the rooting beds filled with wet sand used as rooting substrate after it had been boiled with a view of reducing disease risks.

The experimental design adopted corresponded to a full factorial of (i) the age of the donor plant (2 classes); (ii) the within-shoot position of the cuttings (8 classes) and (iii) the auxin treatment (2 classes), resulting in a total of 2 x 8 x 2 = 32 combinations, each being represented by an experimental plot of 10 totally randomized cuttings. Three complete blocks of this type were set up applying rigorously the same procedure on the following dates and under the same equatorial humid climatic conditions:

— 1st block on the 24th of April 1992;
— 2nd block on the 22nd of May 1992;
— 3rd block on the 23rd of June 1992.

To sum up, the whole experiment included:
10 x 2 x 8 x 2 x 3 = 960 cuttings.

Once set into the rooting bed, the cuttings were maintained under a 50 % shade with intermittent-mist water sprays provided by a mist system, the frequency of which was controlled by the "electronic leaf" system (HARTMANN et al., 1990) to avoid any desiccation damage. Aqueous fungicide solutions — mainly Thyram 80, 5 g/l — were sprayed on the cuttings once a week.

After a rooting period of 2.5 months in these conditions, the cuttings were assessed by recording the following criteria:

1. rooting rate for cuttings (RRC), based on the 10-cutting plots;
2. number of adventitious roots per rooted cutting;
3. root score, defined as the variation coefficient of 3 values corresponding to the number of newformed roots in each axial third of the cross-sectional area of each rooted cutting; thus, the lower the root score, the more even the root distribution around the cutting axis;
4. length of the longest root (in cm) per rooted cutting.

The data were analyzed using the SAS statistical package (SAS Institute Inc., 1988). The null hypotheses were rejected when probability value $P \leq 0.050$. Tests for homogeneity of variance were performed using BARTLETT's test (SNEDECOR and COCHRAN, 1957) and LEVENE's test (TOMASSONE et al., 1983) which both established that the computed variances of RRC were not homogenous enough when referring to classes within each factor, especially regarding the age of the donor plant ($P \leq 0.001$). For this reason RRC was suitably replaced by ASRRCC according to the following angular transformation (SNEDECOR and COCHRAN, 1957): $ASRRC = 2 \arcsin \sqrt{RRC}$.

The effects of the experimental factors on the ASRRC values were evaluated carrying out an analysis of variance according to the following statistical model:

$$Y_{ijkl} = \mu + A_i + P_j + H_k + B_l + (AP)_{ij} + (AH)_{ik} + (PH)_{jk} + (APH)_{ijk} + \varepsilon_{ijkl}$$

where:

Y_{ijkl} = value of ASRRC of the plot submitted to the i^{th} level of factor age of the donor plant, j^{th} level of factor within-shoot position, k^{th} level of factor auxin in the l^{th} block;
μ = overall mean of the trial;
A_i = effect of the factor age of the donor plant, $1 \leq i \leq 2$;
P_j = effect of the factor within-shoot position, $1 \leq j \leq 8$;
H_k = effect of the factor auxin, $1 \leq k \leq 2$;
B_l = effect of blocks, $1 \leq l \leq 3$;
$(AP)_{ij}$ = effect of the interaction of age and within-shoot position;
$(AH)_{ik}$ = effect of the interaction of age and auxin;
$(PH)_{jk}$ = effect of the interaction of within-shoot position and auxin;
$(APH)_{ijk}$ = effect of the interaction of age, within-shoot position and auxin;
ε_{ijkl} = random error.

Deficiency in the ability to form adventitious roots resulted in a large number of missing values especially for the cuttings taken from the lower positions within the shoot. For this reason, the statistical analyses for the

Table 1. — Mean values of the 4 characters assessed for each experimental factor investigated. Letters distinguish means which are significantly different at the 5% level. Data in brackets were not submitted to the analyses of variance and covariance and must be considered as informative only.

CHARACTERS	RRC*1* (%)		No of plots*6*	No of roots*2*		Score*3*		Length*4* (cm)		No of plots
FACTORS	mean	σ*5*		mean	σ	mean	σ	mean	σ	
BLOCK										
1	27.2	27.3	32	2.3	0.7	0.78	0.33	10.0*ab*	2.5	11
2	28.8	29.4	32	2.5	1.4	0.85	0.45	11.7 *a*	3.1	12
3	28.1	30.6	32	2.1	0.7	0.88	0.37	7.5 *b*	1.9	10
DONOR PLANT										
Seedlings	41.5 *a*	30.6	48	2.2	1.0	0.88	0.26	9.7	2.4	18
Stump	14.6 *b*	19.3	48	2.4	1.0	0.79	0.50	10.1	3.7	15
WITHIN-SHOOT POSITION										
1	59.2 *a*	26.8	12	3.0 *a*	1.0	0.60	0.32	9.5	2.0	12
2	45.0*ab*	26.1	12	2.0*ab*	0.8	1.00	0.28	10.3	4.0	11
3	30.8*bc*	25.4	12	1.8 *b*	0.7	0.94	0.41	9.9	3.1	10
4	22.5*cd*	21.8	12	(2.0)	(1.2)	(1.00)	(0.37)	(9.9)	(2.9)	(9)
5	20.8*cd*	23.5	12	(2.3)	(0.9)	(0.98)	(0.27)	(10.9)	(2.7)	(8)
6	22.5*cd*	33.1	12	(1.5)	(0.9)	(1.27)	(0.25)	(8.6)	(2.6)	(7)
7	12.5 *d*	27.0	12	(2.1)	(0.4)	(0.79)	(0.19)	(5.6)	(1.6)	(3)
8	10.8 *d*	13.8	12	(2.0)	(0.7)	(0.79)	(0.51)	(8.1)	(3.8)	(6)
AUXIN										
Control	17.5 *a*	23.7	48	1.9 *a*	0.7	0.92	0.47	9.5	2.3	15
Seradix 3	38.5 *b*	29.9	48	2.7 *b*	1.0	0.77	0.50	10.2	3.6	18
OVERALL SCORES	28.0	28.8	96	2.3	1.0	0.84	0.38	9.9	3.0	33

1: Rate of rooted cuttings.
2: Number of roots per rooted cutting.
3: Root score.
4: Length of the longest root per rooted cutting.
5: Standard deviation.
6: Number of elementary plots; initially every elementary plot included 10-cuttings.

three characters of the newly formed root system were performed only for the cuttings originating from the three upper within-shoot positions. The same analysis of variance model as for ASRRC was used for the number of adventitious roots and the length of the longest root, whilst the root score was submitted to an analysis of covariance with the number of roots as covariable due to the high correlation existing between these 2 variables. The relevant model was:

$$Y'_{ijkl} = \mu + A_i + P_j + H_k + B_l + (AP)_{ij} + (AH)_{ik} + (PH)_{jk} + (APH)_{ijk} + \beta\,(X_{ijkl} - \bar{X}_{....}) + \varepsilon_{ijkl}$$

where μ, A_i, P_j, H_k, B_l, $(AP)_{ij}$, $(AH)_{ik}$, $(PH)_{jk}$, $(APH)'_{ijk}$, ε_{ijkl} as previously defined and in addition:

β = the true common slope of the regression lines;
X_{ijkl} = root number value concomitant to Y_{ijkl};
$\bar{X}_{....}$ = overall average of the root number covariable.

Treatment means were compared using Student-Newman and Keuls test when in F-tests the null hypothesis was rejected ($P \leq 0.05$).

Pearson's correlation coefficients between the 4 variables, RRC being replaced by ASRRC, were calculated computing the data from plots including at least one rooted cutting, that is to say in 66 cases out of 96.

Results

General outlines

At the end of the 2.5 month rooting period, 269 cuttings were found rooted out of the total of 960 initially set, which corresponded to an overall rooting rate of 28 %. Among the unrooted cuttings, very few remained alive (15 out of 691). The highest rooting rate of 80 % (24/30) over the 3 dates ("blocks") was obtained for cuttings originating from the second within-shoot position of the seedlings and treated with Seradix 3. The lowest rooting rates (0 %) corresponded in most cases to experimental combinations involving cuttings from the base of sprouts from the stump. *Table 1* presents the mean values of the various characters assessed for each experimental factor.

Table 2. — **Analyses of variance for the rooting rate (expressed by ASRRC), the number of roots and the length of the longest root per rooted cutting, and analysis of covariance with the number of roots as covariable for the root score.**

	ASRRC			No of roots			Length		Score	
Source	DF[1]	MS[2]	F[3]	DF	MS	F	MS	F	MS	F
Block	2	0.005	0.0	2	1.058	2.6	48.836	5.7*	0.040	0.8
Donor plant (D)	1	14.675	58.5**	1	0.062	0.2	0.000	0.0	0.000	0.0
Within-shoot position (P)	7	2.942	11.7**	2	5.624	13.9**	0.694	0.1	0.037	0.7
Auxin (A)	1	8.815	35.1**	1	8.293	20.5**	8.638	1.0	0.019	0.4
D x P	7	0.234	0.9	2	1.298	3.2	1.559	0.2	0.034	0.7
D x A	1	1.742	6.9*	1	0.019	0.1	3.216	0.4	0.002	0.0
P x A	7	0.290	1.2	2	0.197	0.5	2.691	0.3	0.102	2.0
D x P x A	7	0.120	0.5	2	0.754	1.9	5.065	0.6	0.105	2.1
No of roots				1					0.685	13.4**

1) Degrees of freedom.
2) Mean square.
3) Value of FISCHER's statistical test with significance levels: *: $p \leq 0.05$; **: $p \leq 0.01$.

Correlations

Analysis of correlations between traits showed that the rooting rate might be slightly positively correlated with the average number of newformed roots ($r = + 0.232$, $P = 0.0603$), this latter criterion being strongly negatively correlated with the average root score ($r = - 0.810$, $P < 0.01$).

Analysis of variance, covariance and comparison of means

The analysis of variance summarized in *table 2* shows significant effects of the 3 experimental factors investigated ($P < 0.0001$) and of the „donor plant x auxin" interaction ($P = 0.01$) on the rooting rate, whereas the number of roots was found to be influenced by the within-shoot position and the auxin treatment ($P < 0.0001$). The length of the longest root was observed to be influenced only by the date the different blocks were set ($P = 0.0113$).

To summarize, the following results were obtained (see *Table 1* for accurate data):

1. Cuttings taken from seedlings exhibited higher rooting rates than those from stump sprouts of a 6-year-old ortet.

2. There was a decrease of rooting potential of cuttings from the top to the base of the shoot they had been collected from. This was more pronounced for the mature material as illustrated in *figure 1*. STUDENT-NEWMAN and KEULS' multiple range test confirmed that the terminal shoot cuttings (position 1) rooted overall in greater proportions than those located underneath, exhibiting in ad-

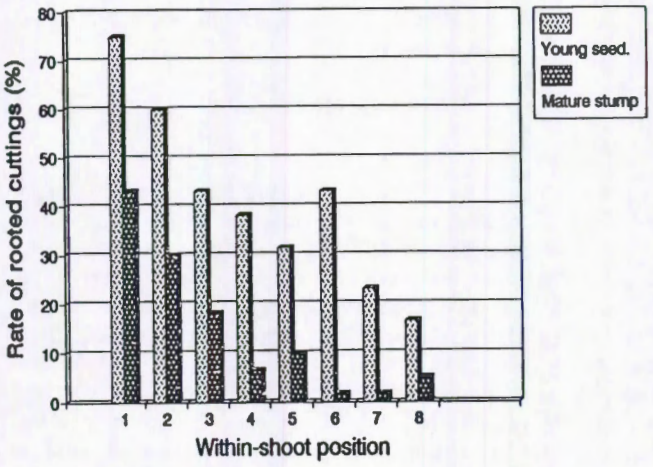

Figure 1. — **Within-shoot position effect on the rooting rate for cuttings (RRC) collected from young seedlings ("Young seed.") and a mature stump of *Acacia mangium*.**

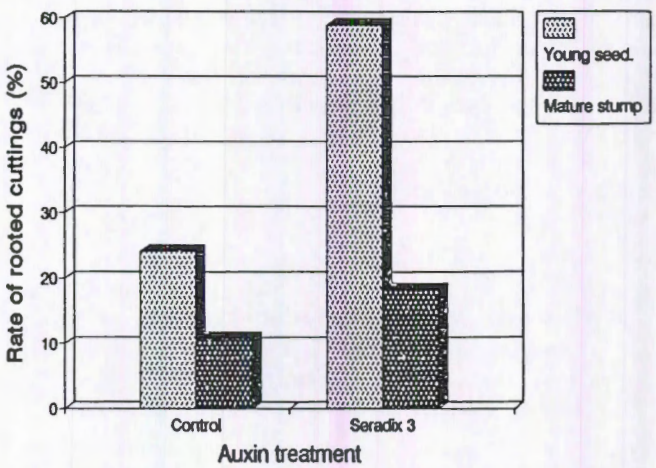

Figure 2. — **Auxin treatment effect on the rooting rate for cuttings (RRC) collected from young seedlings ("Young seed.") and a mature stump of *Acacia mangium*.**

dition more roots and lower root scores than cuttings taken from positions 2, 3, 4 and 6.

3. Cuttings treated with Seradix 3 were shown to root better, producing more roots than the untreated ones.

4. Seradix 3 was more effective on cuttings taken from seedlings than on those removed from stump sprouts, illustrated in *figure 2*.

Discussion

The results obtained from this experiment established that the overall capacity for adventitions root formation by *Acacia mangium* cuttings could be significantly influenced by 3 factors.

The age of the donor plant has in fact been often reported to act negatively on the ability of cuttings to form adventitious roots (Bonga, 1982; Hackett, 1985). In *Acacia mangium*, negative maturation effects on the capacity of ortets to be vegetatively propagated through rooted cuttings seem to appear very early (Darus, 1991). The experiment reported here demonstrated that even sprouting shoots from a mature stump close to the root system, usually considered to be the most responsive plant material (Bonga, 1982; Hackett, 1985), showed much lower rooting rates than juvenile 6-month-old seedlings. The latter exhibited a weaker overall potential for adventitious rooting than the 56-day-old *Acacia mangium* seedlings tested by Wong (1989) in similar experimental conditions, which further demonstrates the negative influence of maturation on rooting ability, in agreement with the findings of Haines et al. (1991). According to Hackett (1988), the difference in terms of rooting rates established between the 6-month-old seedlings and the mature stump sprouts might be due to a deficiency in endogenous promoters or an excess of inhibitors of adventitious root induction in the mature plant material. This is supported by the fact that once rooted, the cuttings displayed similar root system characteristics, regardless of the age of the donor plant. Rooted cuttings of *Acacia mangium* seem to give rise to only a limited number of roots ranging from 2 to 3 on average, even in the case of very young seedlings (Wong, 1989). This could account for the strong correlation found between the number of roots and the root score (*Table 2*). Further investigations are needed to check possible detrimental impact of so few adventitious roots on the quality of the plantations.

Keeping in mind the growth regulator influences, it is not surprising that the cuttings from the top of the shoot, close to the terminal bud where endogenous auxin is assumed to be synthesized (Chaussat and Courduroux, 1980; Hartmann et al., 1990), demonstrated greater capacity for adventitious rooting than those from lower positions. This result however is not consistent with the observation on 56-day-old *Acacia mangium* seedlings where nodal cuttings corresponding to positions 3 to 5 gave the best rooting rates (Wong, 1989). In that latter case, it might be hypothesized that extreme tenderness of the terminal shoot cuttings was responsible for early mortality due to irreparable hydric stress. Another hypothesis related to this within-shoot basipetal decreasing gradient of rooting ability could be associated with anatomical and histological features. The newly formed tissues from the upper part of the shoot may be more prone to form adventitious roots than tissues from lower parts of the shoot that are more differentiated and ontogenetically older, in which the cylinder of sclerenchymatous cells reported by Darus (1989) may constitute and obstacle to root formation and further development.

The beneficial effect of treating the base of the cuttings with "Seradix 3", an exogenous auxin, to improve the rooting rate as well as the quality of the adventitious root system is in agreement with the observations of several authors (Darus, 1989; Wong, 1989). More interesting is the interaction between the auxin treatment and the age of the donor plant. Several hypotheses can be taken into consideration, such as better receptivity of juvenile tissues to the synthetic auxin applied, or major differences between juvenile and mature plant material in endogenous factors involved in adventitious root formation, as reviewed by Hackett (1988).

Conclusion

The results obtained in this experiment tend to confirm that the overall ability of *Acacia mangium* to be propagated by rooted cuttings remains rather limited, especially from mature selected ortets. Furthermore, mature selected *Acacia mangium* may fail to sprout from the stumps once they have been felled. This restricted potential for adventitious root formation has to be fully taken into consideration in tree improvement and development programmes for *Acacia mangium*.

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Structure and Function
Trees

Influence of phase change within a 90-year-old *Sequoia sempervirens* on its in vitro organogenic capacity and protein patterns

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Abstract. Shoot-tips collected from stump shoots at the base of a 90-year-old *Sequoia sempervirens* and from the crown of the same donor tree were compared in respect of their organogenic performance in in vitro conditions. The cultures derived from stump shoots appeared to be much more responsive in terms of growth increment and adventitious rooting capacity – rooting rate, root length, average number of neoformed roots and root score – than cultures derived from the crown, although considerable variation was observed in all the organogenic criteria examined. The persisting topophysical differences in potential for in vitro organogenic and associated morphological traits characteristic of phase change were found to be connected with quantitative modifications of protein content. Thus, 23 membrane-associated proteins with molecular weights ranging from 22 to 58 kDa appeared to be synthesized in greater abundance in the culture originating from the stump shoots.

Key words: Phase change – Protein – *Sequoia sempervirens* – Tissue culture – Topophysical influence

Introduction

The ontogenetic development of woody plants from the juvenile to the mature phase is associated with a number of changes, as reflected by modification of morphology, such as leaf form, or the attainment of the flowering state (Franclet 1985; Hackett 1985). This general phenomenon has been referred to as "ontogenetical ageing" by Fortanier and Jonkers (1976), or as "phase change", which remains the most frequently used terminology (Brink 1962; Hackett 1983, 1985; Wareing 1987).

In arborescent species, it has been stated (Bonga 1982) that the basal parts close to the root system exhibit more juvenile characteristics than upper parts, which are chronologically younger, but appear older from the "ontogenetical ageing" point of view (Hackett 1985). Such a gradient of increasing maturity from the bottom to the top of the tree (Fortanier and Jonkers 1976) is clearly demonstrated in species capable of sprouting from the base of the trunk, where the stump shoots exhibit juvenile features that contrast with the mature characteristics of the shoots located in the crown (Franclet 1985).

The impact of phase change on intraclonal variability has been extensively reported (Hackett 1983), mostly from the standpoint of the negative effects of maturation on physiological abilities for true-to-type cloning, as manifested by difficulties for adventitious rooting or plagiotropic growth habit of the shoots (Hackett 1985). However, so far little is known about the basic origin of such manifestations.

Among the various endogenous compounds susceptible to such investigation, proteins have been the subject of special attention, with studies of their quantitative and qualitative differences in relation to maturity in several species (Fukasawa 1966; Bon 1988a; Bon and Monteuuis 1991; Snowball et al. 1991; Huang et al. 1992). Particular reference has been made to the 16 kDa membrane-associated protein called "J16", which characterized juvenility and connected cloning ability in *Sequoiadendron giganteum* (Bon 1988b). This incited us to concentrate on polypeptides for investigating the phenomenon of phase change and its consequences in terms of organogenic capacity with regard to the location of the shoots within a 90-year-old *Sequoia sempervirens* (D. Don) Endl. This species was selected since: (1) it produces stump shoots; (2) phase change can be easily characterized by salient morphological markers (Franclet 1985); and (3) it is one of the few conifers to react well to in vitro culture conditions.

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Materials and methods

Shoot origins

The *S. sempervirens* from which the shoots were removed was 90 years old, about 30 m in height, and growing at the Arboretum National des Barres, Nogent-sur-Vernisson, France. The shoots consisted of the terminal parts of 0.2–0.8 m long stump shoots arising naturally from the base of the tree, and of shoots located 15 m higher in the crown.

In vitro culture procedure

Material from both origins was systematically paired during all the culture procedures. Cultures were initiated using 0.5 cm shoot-tips in accordance with Verschoore-Martouzet (1985). At the time of the experiments, the 2-cm-long microcuttings consisting of the upper part of the shoot with its terminal bud had been subcultured for 2 years at 2-month intervals exclusively on the elongation medium (EM) that derived from a half-strength Murashige and Skoog (MS) medium supplemented with 20 g l^{-1} activated charcoal (Boulay 1977; Fouret et al. 1986). The rooting procedure was described by Fouret et al. (1986): shoot-tips, 2 cm long, were induced for rhizogenesis for 1 week on a rooting induction medium (RIM) containing 5.10^{-5}M NAA, then transferred for 6 weeks to the same medium without NAA, called rooting elongation medium (REM). All these media, except RIM, lacked growth regulators. The cultures were maintained under a 16 h photoperiod with a light intensity of 60–70 $\mu Em^{-2}s^{-1}$ provided by "Mazda-Fluor Lumière du Jour" fluorescent lamps, except for the rooting phase, which was carried out in darkness. Other experimental conditions were according to Fouret et al. (1986).

Evaluation methods of organogenic potentiality

Shoot growth was evaluated by measuring shoot increment at the end of the three successive 2-month subcultures on EM medium. Each sampling involved at least 50 individuals. Capacity for rhizogenesis was examined at the end of the 6 weeks on REM. Additionally to the proportion (%) of rooted microcuttings, the following traits were recorded for each rooted explant: (1) total number of roots; (2) measurement of the longest root; (3) a root score, which permits the quantitative evaluation of the spatial distribution of the adventitiously neoformed roots. This last criterion was applied as defined by Struve and McKeand (1990): "dividing the cross-sectional area of each rooted microcutting base into quadrants. the longest root in quadrant 1 was assigned 1 point toward the root score; any additional roots in quadrant 1 were each assigned 0.25 points. whereas those in quadrants 2 or 4 were assigned 0.5 points and those in quadrant 3 were assigned 0.75 points". Rooting experiments were repeated 4 times. Each date × origin combination included 20–30 explants.

Using SAS software (SAS 1987), data were subjected to the χ^2-Pearson's test or the analysis of variance test (F test) to determine significant differences ($P < 0.05$ level of probability) among the experimental treatments.

Biochemical assays

Sample preparation and protein extraction. Protein analyses were carried out on elongating microcuttings on EM medium at the end of the two 2-month subcultures. Five hundred milligrams (fresh weight) of each material origin, corresponding to an average of 10 shoots, was extracted in the following cooled buffer: 100 mM TRIS-HCl (pH 8.2), 1 mM Na_2 EDTA, 2 mM $MgCl_2$, 0.25 M sucrose, β-mercaptoethanol 1% and 50 $\mu g.ml^{-1}$ phenylmethylsulphonyl fluoride (PMSF). The homogenate was then filtered through four layers of cheesecloth, centrifuged at 5000 g for 10 min and the resulting pellet discarded. The supernatant was centrifuged at 34000 g for 1 h to obtain a pellet, which was rinsed with TRIS buffer before being centrifuged at 34000 g for 1 h. The resulting pellet was resuspended in TRIS buffer containing 0.5% deoxycholate, homogenized vigorously and stored at 4°C overnight. The homogenate was centrifuged at 34000 g for 1 h to yield a microsomal protein-enriched supernatant. Proteins of this fraction were quantified according to the method of Bradford (1976) using bovine serum albumin as the standard. This microsomal fraction was treated with cooled acetone and β-mercaptoethanol 0.07%, dried under vacuum before the precipitated proteins were solubilized according to Granier and de Vienne (1986) in UKS buffer containing 9.3 M urea, 5 mM K_2CO_3, 0.5% SDS, 4% Triton X-100 and 5% β-mercaptoethanol.

Gel electrophoresis. Two-dimensional separation of proteins was adapted from Bon (1989) with the following modifications: the dimensions of the glass tubes were 200 mm long and 1 mm internal diameter; the isoelectric focusing was carried out at 10000 V × h, and the gels of the second dimension were run at a constant current of 30 mA.

Proteins were stained with silver nitrate in accordance with Bon (1989) immediately after electrophoresis.

Four gels, two for each of two consecutive subcultures on EM, were analysed for each of the two material origins.

Results

Capacity for organogenesis

It appears from the three growth evaluations (Fig. 1 a) that the microcuttings from the basal part of the tree (stump shoots) exhibited higher growth increment than those from the crown (55.6 mm vs 39.6 mm on average, $P < 0.0001$). Growth performances in in vitro conditions were found to be greatly influenced by time course ($P < 0.0001$), with a strong origin × date interaction ($P < 0.0001$), as reflected by more or less pronounced differences between the two origins relative to the dates. Concurrently, original morphological features persisted, with much more developed leaves for the stump shoots than from the crown (Fig. 1 f).

Referring to the ability for adventitious rooting, the basal part of the tree was overall more responsive than the crown in terms of rooted microcuttings (75% vs 19%, $P < 0.001$), as well as when considering the average number of roots per rooted explant (3.6 vs 1.7, $P = 0.006$), the average measurement of the longest roots (54.4 mm vs 42.3 mm, $P = 0.0151$) and the average root score (2.3 vs 1.3, $P = 0.0003$). Whatever the trait examined, the scores for each origin were susceptible to great fluctuations related to the date of the experiment ($P < 0.002$), as illustrated in Fig. 1 b–e.

Protein patterns

The two-dimensional patterns of the microsomal protein fraction showed that about 450 spots could be routinely resolved for each shoot origin (Fig. 2 a–b), with quantitative differences in relative spot size and staining intensity. For the following results, our attention was focused on the spots scored as variable between the origins but unvaried during the time course. The spots which were susceptible to differ between the two dates of collection should be recorded in future experiments, where a greater number of subcultures are considered.

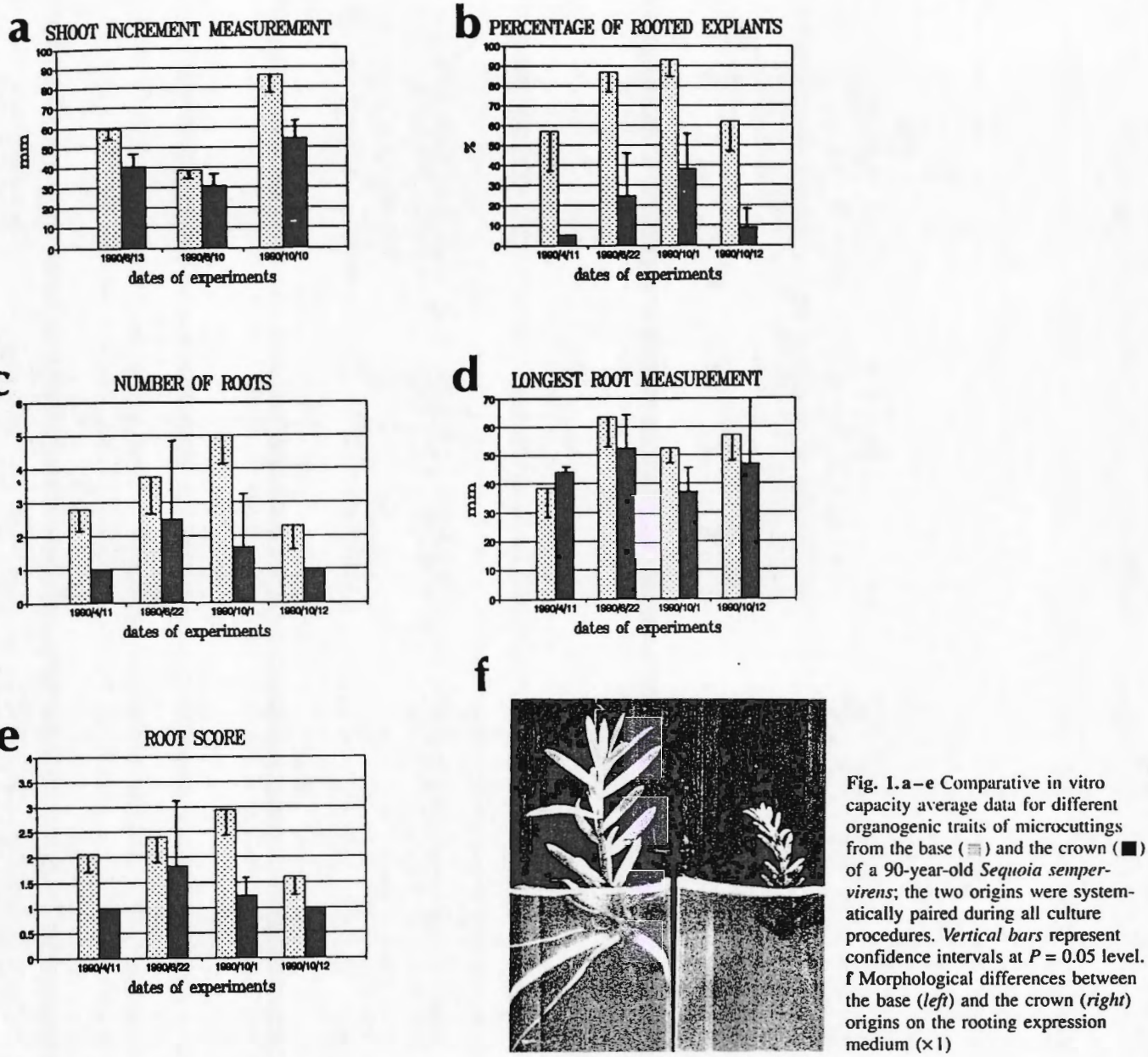

Fig. 1.a–e Comparative in vitro capacity average data for different organogenic traits of microcuttings from the base (▒) and the crown (■) of a 90-year-old *Sequoia sempervirens*; the two origins were systematically paired during all culture procedures. *Vertical bars* represent confidence intervals at $P = 0.05$ level. **f** Morphological differences between the base (*left*) and the crown (*right*) origins on the rooting expression medium (×1)

Three clusters of spots appeared in greater quantity in basal shoot origin than in the crown one (Fig. 2a). The first cluster was constituted of proteins of apparent 55–58000 molecular weight, approximately 33000 molecular weight for those composing the second cluster, and reduced to 22–24000 molecular weight for the third cluster. Proteins 1–5 in the first cluster, 6–14 in the second cluster and 15–23 in the third cluster were detected at high relative rates in the stump shoot origin as compared to the crown one. Whatever the date of collection examined for the basal origin, a subset composed of polypeptides 2, 3, 15, 16, and 20–23 seemed the most representative of the protein differences between the two origins (Fig. 2b). Our experiments did not reveal any qualitative difference in terms of the presence or absence of polypeptides between the samples collected from the stump or from the crown.

Discussion

The reported results attest the salient influence of the in situ location of the shoots within the donor tree on their subsequent potential for in vitro organogenesis in respect to shoot elongation and adventitious rooting ability, despite noticeable fluctuations during time course that might be due to endogenous rhythm effects (Champagnat et al. 1986). The results support the hypothesis that in trees, the intensity of the maturation process is susceptible to vary according to an intra-individual zonation, which generally increases from the bottom to the top (Bonga 1982). Similar trends were observed by Sanchez and Vieitez (1991) with regard to in vitro performance of basal and crown shoots in mature chestnut. The morphological and organogenic differences pointed out in our study were shown to have been

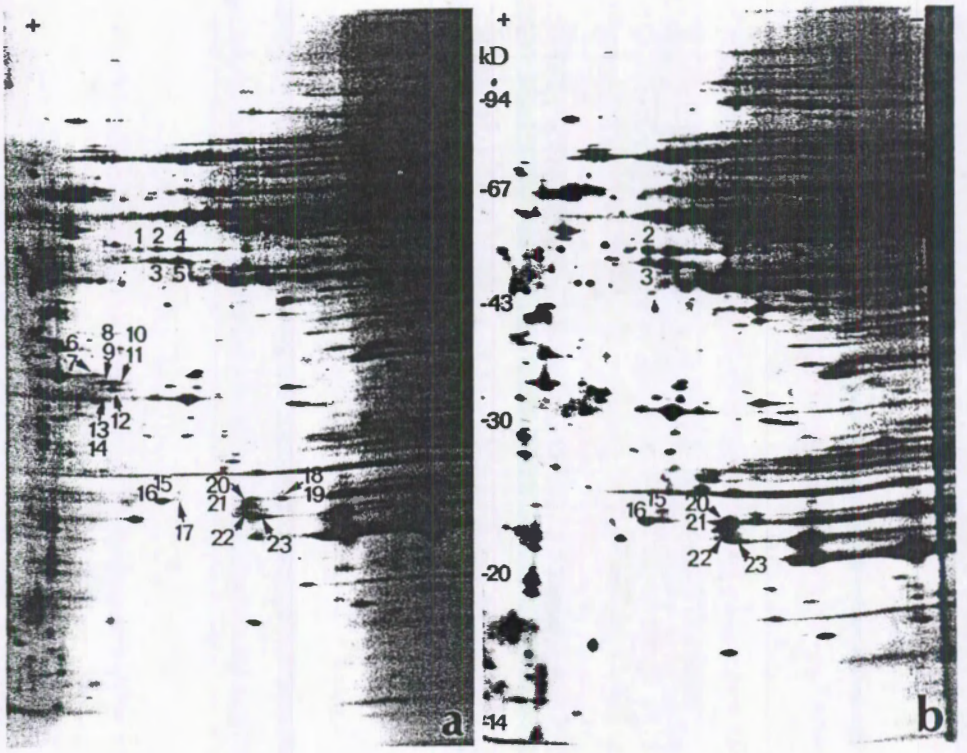

Fig. 2. Silver-stained two-dimensional gel of microsomal proteins extracted from microcuttings originated from the crown (**a**) and from the base (**b**) of a 90-year-old *Sequoia sempervirens*. In **a**, the *arrows* and the corresponding *numbers* indicate the quantitative differences of proteins with the basal origin. Eight of these 23 different proteins, significantly in higher abundance in the microcuttings originated from the base, are indicated in **b**

maintained for 2 years in the experimental in vitro culture conditions. The persistence of this phenomenon, which was referred to as topophysis by Dodd and Power (1988), accounts for the intra-clonal variability so frequently noticed when attempting to clone mature trees.

Studies have been carried out on the same species to ascertain whether it is possible to reverse the phase change phenomenon, especially by subculturing in vitro the explants on cytokinin-enriched media (Fouret et al. 1986). This accounted for the positive effect of exogenous cytokinins to revert, at least temporarily, some mature traits, and is consistent with the hypothesis that the basal part of trees remains more juvenile due to natural root-produced cytokinins (Van Staden and Harty 1988). However, the fact that the presumed rejuvenation did not persist outside of its special experimental environment (Fouret 1987) illustrated once more the basic determinism of phase change.

The topophysis aspects observed were found to be associated with modifications of the microsomal protein fraction for which some polypeptides were found to be more abundant in stump shoot tissues that were presumably more juvenile. In *Sequoiadendron giganteum*, the rejuvenated line as well as the juvenile control also exhibited higher overall polypeptide concentrations than the original mature material, which was much less responsive for organogenesis (Monteuuis 1988). The prevailing trend emerging from the various but still quite limited investigations carried out in this field so far is that phase change involves both quantitative and qualitative changes, in terms of presence or absence of polypeptides, in protein contents (Huang et al. 1992), with a specific protein for the mature *Citrus* (Snowball et al. 1991), or with a greater number of spots in the more juvenile material (Bon and Monteuuis 1991). Special care has to be taken when examining grafted material. as shown by Bon and Monteuuis (1987) working on shoots from grafted meristems collected from a 100-year-old giant sequoia, and who observed additional polypeptides, especially acidic ones, and some more abundant spots in the scions exhibiting mature traits as compared to the temporary morphologically rejuvenated ones. As hypothesized by Huang et al. (1992) grafting may involve the transfer of small molecules from the rootstock that might interfere with the scion peptides. In *S. giganteum*, the fact that specific immunoblotting against "J16" polypeptide revealed a second band only in tissue extracts from meristem grafts (Bon 1988b) tends to support this hypothesis.

Among the 23 microsomal proteins which appeared to be more abundantly synthesized in the material from the base, the results obtained so far, however, do not allow the identification of any of them to distinguish reliably between the two "topoclones", as was done with the so-called "J16" to differentiate between the juvenile and the mature status in *S. giganteum* whatever the environmental conditions (Bon 1988b). Also further investigations carried out to consider separately the total and the soluble protein fractions failed to establish any significant difference between the two origins. All these protein fractions were shown to be influenced too much by the physiological status which fluctuates greatly during the time course for each plant material origin, as illustrated by the reported variations of organogenic capacity. This is the reason why emphasis was given to the microsomal fraction, shown to be less affected by uncontrolled fluctuations of physiological status, and hence more reliable in terms of phase change indicators. Additional experiments are needed, however, to obtain a better evaluation of the date interaction on the microsomal protein patterns. Fluctuating physiological status is indeed a

disturbing parameter which needs to be fully taken into consideration when investigating phase change in trees (Monteuuis and Bon 1986), even for plant material from the same genotype.

Lastly, the importance of carring out basic biochemical and molecular studies of phase change on plant material from the same genotype in order to avoid distorting genotypic interactions has to be emphasized. In that respect, *Sequoia sempervirens* with the coexistence within the same individual of juvenile and mature forms constitutes a good model to further our knowledge about the epigenetic basis of phase change.

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In vitro rooting of micropropagated shoots from juvenile and mature *Pinus pinaster* explants: influence of activated charcoal

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Abstract

The ability for adventitious rooting of micropropagated shoots from juvenile and mature *Pinus pinaster* Ait. explants was assessed in vitro on a rooting expression medium. The different rooting traits observed, namely the rooting rate, the number and the length of the adventitious roots, and the root score, were greatly influenced by the age of the donor plant: 98% of juvenile explants rooted, while only 49% of mature explants did. Addition of activated charcoal in the rooting expression medium improved the overall rooting capacity of the mature explants to an average of 78%. Whatever the plant material, the number and the length of the adventitious roots, as well as the root score, fluctuated according to the sampling date.

Abbreviations: BA – 6-benzyladenine, NAA – naphthaleneacetic acid, REM – rooting expression medium, RIM – rooting induction medium

Introduction

Although the benefits of using tissue culture for forest trees have been recognized for many years (Timmis et al. 1987; Thorpe et al. 1991), the success of such methods are still highly dependent upon the ability to acclimatize the rooted micropropagated shoots (Mohammed & Vidaver 1988). This is particularly obvious for coniferous species, for which rooting of tissue culture-produced plantlets is still problematic, especially in the case of mature plant material (Thorpe & Biondi 1984), despite the good progress made in *Pinus radiata* (Horgan & Holland 1989). When cultivated in vitro, *Pinus pinaster* Ait., an economically important coniferous species for many temperate countries, reacts consistently with this tendency. Furthermore, despite the fact that it has been possible to propagate mature selected clones in vitro by axillary shoots (Dumas 1987), rooting of the produced microcuttings requires further improvement in order to take full advantage of this promising technology (Chaperon 1991).

Among the numerous substances available to improve in vitro rooting of micropropagated shoots (McCown 1988), activated charcoal deserves special consideration owing to its reported positive influence in stimulating the rooting potential of explants from various species and from pines in particular (Patel & Thorpe 1984). For this reason, activated charcoal was selected as a priority factor to analyze to what extent it could improve the in vitro rootability of juvenile and mature micropropagated shoots of *Pinus pinaster*.

Materials and methods

Plant material and in vitro culture conditions

Pinus pinaster explants of two different ages were used. Explants of mature material were 2- to 3-cm-long apical segments of elongating shoots from a 17-

year-old superior genotype established in vitro using Dumas' (1987) procedure. Explants of juvenile material were from seedlings germinated in vitro in accordance with Dumas et al. (1989). These two explant materials, systematically paired during all culture procedures, were propagated for 1 year with alternating subcultures of 3 weeks on multiplication medium with 10^{-5}M BA plus 5.10^{-8}M NAA – and 1 month on an activated charcoal-enriched (20 g l^{-1}) elongation medium described by Dumas (1987). Microcuttings 3- to 4-cm-long with an apical bud, obtained from shoots developed on the elongation medium, were used for rooting. These microcuttings were first placed on a rooting induction medium (RIM) derived from Rancillac et al. (1982) that consisted of full-strength Risser & White (1964) macronutrients, half-strength Murashige and Skoog (1962) micronutrients, 50 mg l^{-1} myo-inositol, 2 mg l^{-1} glycine, 1 mg l^{-1} thiamine, 1 mg l^{-1} pyridoxine-HCl, 1 mg l^{-1} nicotinic acid, 10 g l^{-1} sucrose and 5.4 µM NAA. After pH adjustment to 5.5–5.6, 8 g l^{-1} agar (K267, Kadoya) were added and the medium was autoclaved at 110 °C and 69 kPa for 30 min. After 3 weeks, the microcuttings were then transferred onto a rooting expression medium (REM) that differed from RIM only for sucrose concentration (30g l^{-1}) and in totally lacking NAA. The influence of 20g l^{-1} activated charcoal (Merck 2186) added to REM on the rooting capacity of the explants was tested.

All the cultures were placed in glass test tubes (25 × 200 mm) containing 20 ml of culture medium and closed with "Magenta" polypropylene caps and were maintained under a 16-h photoperiod (120 µmol m^{-2} s^{-1}, "Sylvania Cool White" fluorescent lamps 36 W) at 25/22 ± 2 °C light/dark.

Evaluation of rooting ability

The capacity for adventitious rooting on REM was examined at the end of 4 weeks. In addition to determining the percent of rooted microcuttings, the following traits were also determined for each rooted microcutting:

- total number of roots emerging from the microcutting base;
- length of the longest root;
- quantitatively evaluating the spatial distribution of the adventitious roots (root score as defined by Struve & McKeand 1990).

The root score was determined by dividing the cross-sectional area of each rooted microcutting base into quadrants and assigning the longest root in quadrant 1 1 point toward the root score. Any additional roots in quadrant 1 were each assigned 0.25 point, whereas those in quadrants 2 or 4 were assigned 0.5 point and those in quadrant 3 were assigned 0.75 point.

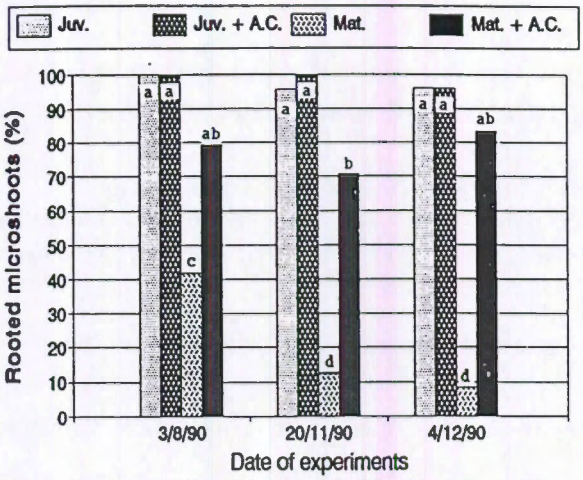

Fig. 1. Percentages of rooted microshoots from juvenile (Juv.) and mature (Mat.) *Pinus pinaster* explant sources tested at different dates in absence or in presence (+ A.C.) of activated charcoal in an in vitro rooting medium. Bar values with different letters indicate significant differences at the 5% level.

Rooting experiments involved 24 microcuttings for each of the four experimental combinations tested (REM with or without activated charcoal × juvenile or mature plant material) and were repeated at three different dates.

Using SAS (SAS Institute, Inc. 1987), the data were subjected to the χ^2-Pearson's test or the analysis of variance test (F test) and to Tukey's multiple comparison test to determine significant differences among the experimental treatments at $p = 0.05$.

Results

Rooting rates

The rooting rate scores summarized in Fig. 1 were shown to be significantly influenced by the age of the explant material ($p < 0.001$) with higher scores for the microshoots derived from the juvenile explants (98% versus 49% for the mature clone, on average). Addition of activated charcoal to REM resulted in a remarkable ($p < 0.001$) enhancement of the rooting percentage for the mature explant material (average rooting rates of 78% vs 21% in absence of activated charcoal), with a

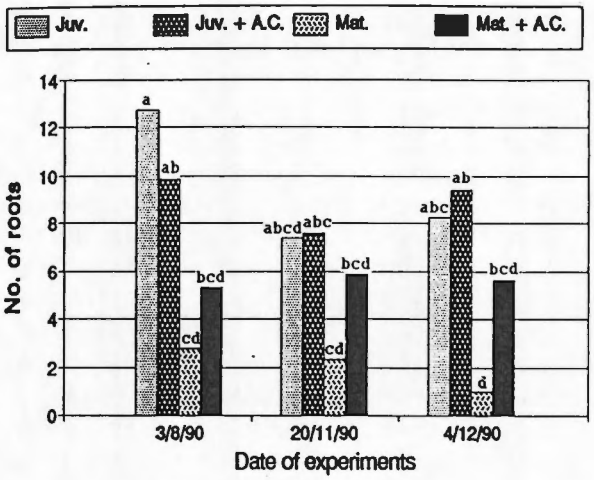

Fig. 2. Average number of adventitious roots produced by rooted microshoots from juvenile (Juv.) and mature (Mat.) *Pinus pinaster* explant sources tested at different dates in absence or in presence (+ A.C.) of activated charcoal in an in vitro rooting medium. Bar values with different letters indicate significant differences at the 5% level.

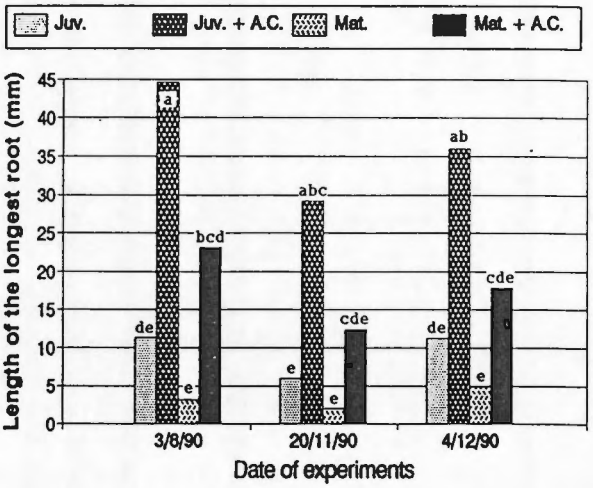

Fig. 3. Average length of the longest adventitious root produced by rooted microshoots from juvenile (Juv.) and mature (Mat.) *Pinus pinaster* explant sources tested at different dates in absence or in presence (+ A.C.) of activated charcoal in an in vitro rooting medium. Bar values with different letters indicate significant differences at the 5% level.

strong age × activated charcoal interaction ($p < 0.001$). The influence of the sampling date on the rooting rates was not significant.

Number of roots

As illustrated in Fig. 2, the rooted microshoots derived from the juvenile explant material produced more roots

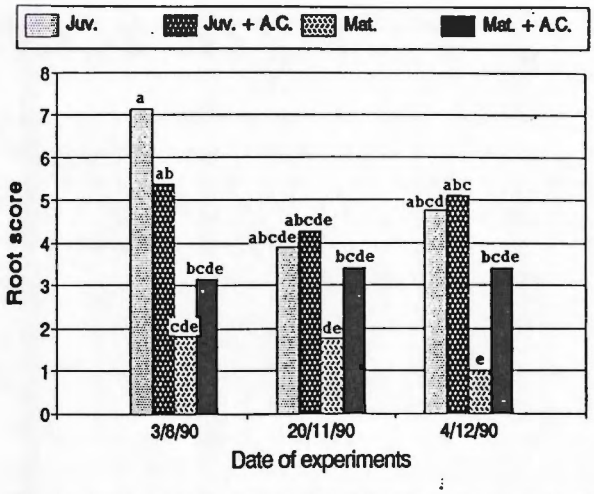

Fig. 4. Average root score (see text for definition) values obtained for rooted microshoots from juvenile (Juv.) and mature (Mat.) *Pinus pinaster* explant sources tested at different dates in absence or in presence (+ A.C.) of activated charcoal in an in vitro rooting medium. Bar values with different letters indicate significant differences at the 5% level.

on average than the ones from mature explants ($p < 0.0001$). However, more roots on the latter resulted in the presence of activated charcoal ($p = 0.0064$). The number of adventitious roots formed varied ($p = 0.0323$) with the sampling date, especially for the juvenile explant material ($p = 0.0277$).

Length of the longest root

The age of the explant material was shown to have a significant ($p < 0.0001$) effect on the length of the longest root produced, with higher scores for the juvenile explants. Microshoots rooted in the presence of activated charcoal also produced the longest roots ($p < 0.0001$), especially in the case of the juvenile explant material ($p = 0.0092$). Root length was affected by the date of the experiment ($p < 0.0001$), with significant differences ($p = 0.0450$) depending on whether the rooting expression medium contained activated charcoal or not (Fig. 3).

Root score

The quality of the root system derived from the juvenile explant material as evaluated by the average root score (Fig. 4) was superior ($p < 0.0001$) to that of the mature explant material, with marked differences ($p = 0.0133$) according to the sampling date. Furthermore, statistical analysis established that variations of

root score from the date of one experiment to another was significantly different for both the juvenile and the mature explant material ($p = 0.0126$). A strong interaction ($p = 0.0045$) between the age of the explant material and activated charcoal was observed. Furthermore, the mature explant microcuttings rooted in the presence of activated charcoal resulted in an increase in the root score, whilst the effect was not as well defined for the juvenile explant material.

Discussion

The fact that the juvenile plant material exhibited an overall greater potential for adventitious rooting than the mature clone is consistent with many reports emphasizing the inhibitory effect of the maturation process on the capacity to form adventitious roots (Hackett 1988; Mohammed & Vidaver 1988). It should be noted, however that the juvenile stock rooting rates were similar to those obtained by Rancillac et al. (1982) for shoots derived from 1-month-old *Pinus pinaster* seedlings. The present study established that rooting rates, number of roots, root length and the root score could be affected by the stage of maturity of the explant source. Concomitant experiments performed with the same conditions and involving explants from different mature genotypes of *Pinus pinaster* confirmed this same tendency, rather than any particular genetic effect (Haissig & Riemenschneider 1988). Similar findings were observed within a 90-year-old *Sequoia sempervirens* tree between shoots from the base of the ortet, physiologically more juvenile, and the ones from the crown, which were more mature and much less responsive to adventitious rooting (Bon et al. 1994). Various endogenous factors presumed to be involved in reduced rooting capacity during maturation have been reviewed by Hackett (1988) with additional data currently expected from molecular approaches (Goldfarb et al. 1992). Mature explant material for instance may react to an inductive signal with reduced potential for biosynthesis, especially protein synthesis, in comparison with the juvenile explant source, as pointed out by Bon (1988) with *Sequoiadendron giganteum*.

Of interest is the positive influence of the activated charcoal on the rootability of the microshoots resulting in a significant increment of the four rooting traits examined and of the shoot development. Rooting rate enhancement in activated charcoal-enriched media has already been reported for different species such as *Allium cepa* (Fridborg & Eriksson 1975) and *Sequoiadendron giganteum* (Monteuuis & Bon 1986). This is especially true for the rooting expression phase in medium lacking auxin, as mentioned for *Pinus contorta* (Patel & Thorpe 1984) and *Pinus sylvestris* (Gronroos & von Arnold 1985). More ambiguous results were observed for activated charcoal incorporated in auxin-enriched rooting induction medium (Bekkaoui et al. 1984; Monteuuis & Bon 1986), perhaps because of the partial adsorption of the exogenous auxin by the activated charcoal (Weatherhead et al. 1979). The positive effect of activated charcoal on the rootability of the microshoots in the rooting expression medium can be interpreted by considering at least two aspects:

- reduction of light at the base of the shoots, thus providing an environment conducive to the accumulation of photosensitive auxin or cofactors (Druart et al. 1982), and
- adsorption of adventitious rooting inhibitors (Fridborg & Eriksson 1975; Fridborg et al. 1978; Misson et al. 1983).

The interacting effect with sucrose or derivatives resulting from autoclaving has been hypothesized also (Misson et al. 1983). According to van Waes (1987), and more specifically Bon et al. (1988) working on juvenile and mature clones of *Sequoiadendron giganteum*, activated charcoal may interfere with polyphenolic compounds produced by the explants, thus modifying the endogenous ratio between the free and the conjugated polyphenols, the influence of which on the organogenic potential including shoot and root development has been long recognized (Druart et al. 1982). The variations noted here of activated charcoal effects on rootability in relation to the age of the micropropagated shoots and to the sampling date could be due to the fact that polyphenol biosynthesis is susceptible to changes in quality and quantity during the time course of the experiment and to differences in the maturity of the explant material, as already established for other plants (Jay-Allemand 1985; Bon 1988).

Conclusion

As with other species (Monteuuis & Bon 1986; Bon et al. 1994), in vitro rootability of *Pinus pinaster* micropropagated shoots in stabilized environmental conditions is subject to variation according to the maturity of the explant material and the sampling date. Addition of activated charcoal in the rooting expression medium was shown to improve the potential for adventitious rooting, not only in terms of rooting rates, but also in

enhancement of the number and the length of the roots, as well as the root score. This stimulating effect of activated charcoal on rootability was particularly associated with mature explant material. Although further investigations are needed for a better understanding of the roles of the activated charcoal on the physiology of the explants in relation to adventitious rooting, its utilization provides for the production of cloned plantlets of selected *Pinus pinaster* genotypes for the greatest profit of tree improvement and plantation programmes.

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Rooting *Acacia mangium* Cuttings of Different Physiological Age with Reference to Leaf Morphology as a Phase Change Marker

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Summary

Capacity for adventitious rooting of *Acacia mangium* was assessed for shoot terminal cuttings originating from: (A) the crown and (B) sprouting stumps of 4-year-old trees growing outdoors, (C) 4-year-old hedged stock plants and (D) 1-year-old seedlings kept cultivated in containers. All these cuttings exhibited the mature phyllode morphology. For the stump sprout origin, rootabilities of juvenile-like composed leaf (B1), intermediate leaf (B2) and mature-like phyllode (B3) cuttings were also compared.

Rooting potential was found to be greatly influenced by the cutting source and to lesser extent, although still significantly, by the different types of cutting morphology. In both cases, the highest average rooting rate score of 85% was obtained for the mature-like phyllodes cuttings derived from mature sprouting stumps, whereas the same origin cuttings with juvenile-like leaves were less prone to root. Exogenous auxin treatment was shown to improve noticeably the number of roots formed per rooted cutting, but had overall no effect on rooting rate with marked differences depending on the date of the experiment.

These results are discussed in terms of ageing influence on the potential for adventitious rooting of *Acacia mangium* cuttings, with reference to leaf morphology as a phase change marker.

Key words: Acacia mangium, age, auxin, cutting, maturation, morphological marker, phase change, rootability, vegetative propagation.

FDC: 161.4; 164.3; 164.5; 165.441; 232.11: 232.411.4; 176.1 *Acacia mangium.*

Introduction

Acacia mangium WILLD. has gained an increasing interest for reforestation programmes in the humid tropics over the last 2 decades mainly for pulpwood production. This is due to the

remarkable growth potential of this pioneer tree legume, even on very acid and infertile soils it can rehabilitate thanks to its natural nitrogen fixing ability. For a few years, special efforts have been devoted on tree improvement of this fast-growing species. Progress in that field is just beginning and although simple breeding strategies based on sexual propagation seem objectively well adapted to the situation (MONTEUUIS and NASI, 1992), the vegetative propagation option is also worth particular consideration. First experiments tended to indicate that while desirable in theory (HAINES and GRIFFIN, 1992), the prospects for propagating vegetatively *A. mangium* through rooted cuttings are rather limited, mostly due to early negative effects of the maturation process on the potential for adventitious rooting of this species (DARUS, 1991; POUPARD *et al.*, 1994).

Decrease or even loss of ability for true-to-type cloning as trees become larger in size with increasing age has been already reported for many species (SCHAFFALITZKY DE MUCKADELL, 1959; BONGA, 1982; HACKETT, 1983, 1985), and the need to find simple markers of this so-called phase change phenomenon in various species to select within the donor plant the more juvenile shoots with greater potential for adventitious rooting, has called for special attention (HACKETT, 1985; MONTEUUIS, 1985). *A. mangium* is a species exhibiting salient differences in leaf morphology associated with the first phases of the ontogenetical process (DOORENBOS, 1965). The first leaves, or "composed leaves", are exclusively composed of pinnates, the number of which increase from one for the first leaf formed to 4 for the 6th or 7th leaf, before the appearance of an intermediate leaf type consisting of a phyllode with 4 and then 2 pinnates attached to its apex, to become a full phyllode from the 9th to 11th node position upward, that corresponds roughly to 12 weeks to 16 weeks after germination depending on the local conditions (RUFELDS, 1988; GAN and SIM, 1992). From that age onwards, *A. mangium* seedlings produce phyllodes exclusively, that characterizes the mature condition (DOORENBOS, 1965). It was the aim to find out to what extent juvenile foliage characteristics can be considered reliable markers of potential for adventitious rooting of cuttings in *A. mangium*. This has been further investigated examining rootability of samples of *A. mangium* shoots coming from different age donor plants or differing in ontogenetical age and leaf morphology, in relation to exogenous auxin treatment, the beneficial influence of which on rooting capacity has been already reported several times (DARUS, 1989; WONG, 1989; WONG and HAINES, 1992; POUPARD *et al.*, 1994).

Material and Methods

Terminal shoot cuttings with an apical bud of *A. mangium* used for rooting experiments were collected from 4 different types of donor plants all from the same Papua-New Guinea – "PNG" – seed provenance. Unless otherwise stated, they were softwood cuttings bearing only phyllodes:

A. lower part of the crown – about 4 m to 5 m above ground level – of 4 year-old *A. mangium* planted trees;

B. 60 cm to 80 cm tall stumps obtained by decapitating 1.5 month earlier some of the above mentioned trees, that resulted in the production of 3 week-old sprouting shoots, 30 cm as maximal length, collected to make cuttings and distinguishing between:

B1: cuttings with composed leaves exclusively;

B2: cuttings with intermediate phyllode-pinnate morphology;

B3: cuttings with phyllodes exclusively;

These 3 types of cuttings, more herbaceous than the other origins, are illustrated in *figure 1*;

C. 4 year-old stock plants kept extensively cultivated potted in 5 l plastic bag containers filled with local top soil and maintained at an height of 50 cm to 60 cm by hedging in the nursery;

D. main stem of 1 year-old seedlings potted in 1 l plastic bag containers filled with local top soil and kept cultivated in the nursery.

The average size of the cuttings was around 6 cm in length from the basal cut to the apical bud whatever the type, with

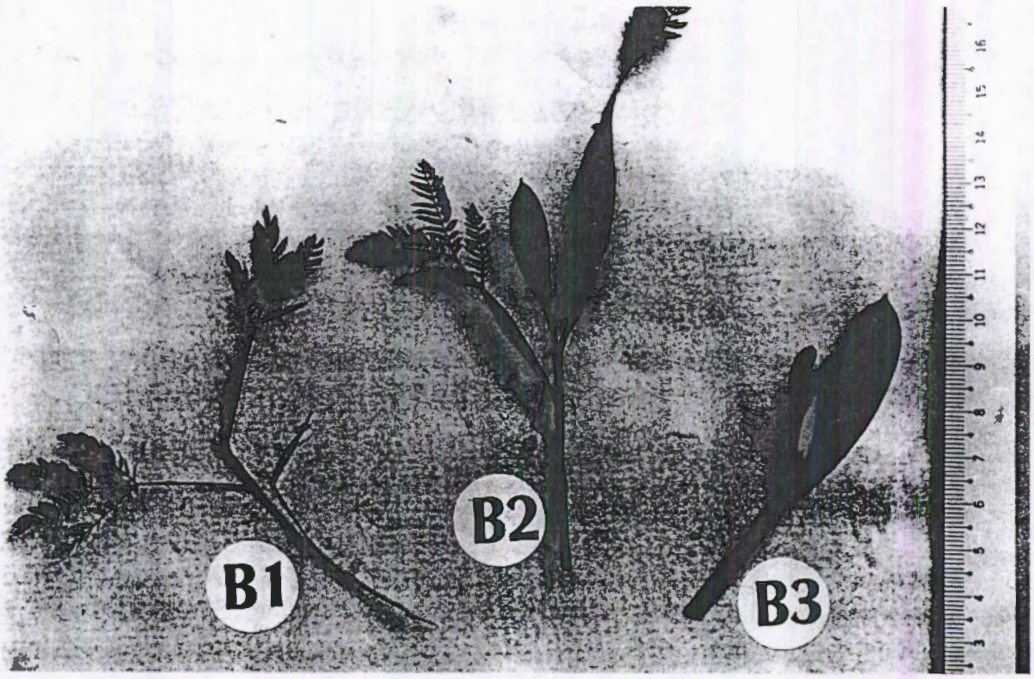

Figure 1. – Distinctive morphological features of composed leaf (B1), intermediate leaf (B2) and phyllode (B3) cuttings produced by the mature stumps and used for the experiments.

smaller diameter for the composed-leaf and intermediate morphology cuttings than for the mature-like ones exclusively with phyllodes, as shown in *figure 1.*

Half of the cuttings corresponding to each of the different categories used were treated with auxin by dipping their base into a SERADIX 3 commercial powder preparation (0.8% of 3-indolebutyric acid in talc), the remaining being treated as controls.

All the cuttings were finally inserted into rooting beds filled with wet sand used as rooting substrate after it had been boiled with a view of reducing disease risks.

The study consisted of 2 distinct sub-experiments – "exp.1" and "exp.2" – set up applying strictly the same procedure and under the same equatorial humid climatic conditions on 2 different dates:

- October 14, 1992 for exp.1;
- May 1, 1994 for exp.2.

The experimental design adopted for each sub-experiment corresponded to a full factorial of (i) the cutting category,

- from A to C, that is to say 5 classes for exp.1;
- and from A to D, that is to say 6 classes for exp.2;

and (ii) the auxin treatment (2 classes), resulting in a total of:

- 5 x 2 = 10 combinations for exp.1;
- and 6 x 2 = 12 combinations for exp.2.

For both sub-experiments, each combination was represented by 3 plots of 10 cuttings randomly distributed within 3 complete blocks.

To sum up, the whole experiment comprised:
(10 x 3 x 5 x 2) + (10 x 3 x 6 x 2) = 660 cuttings.

Once set into the rooting bed, the cuttings were maintained under a 50% shade with intermittent-mist water sprays provided by a mist system, the frequency of which was controlled by an "electronic leaf" system (HARTMANN *et al.*, 1990) to avoid any desiccation damage. Aqueous fungicide solutions – mainly Thyram 80, 5 g/l – were sprayed on the cuttings once a week.

After a rooting period of 2 months in these conditions, the cuttings were assessed by recording the following criteria:

1. *rooting rate for cuttings (RRC),* based on the plots with 10 cuttings each;
2. *number of adventitious roots (NR)* per rooted cutting;
3. *length of the longest root* (in cm) per rooted cutting.

The data were analyzed using the SAS statistical package (SAS Institute Inc., 1988). Null hypotheses were rejected when the probability value was $P \leq 0.050$. Tests for homogeneity of variance were performed using BARTLETT's and LEVENE's tests (SNEDECOR and COCHRAN, 1980) which both established the need of replacing RRC by ASRRC = $\arcsin\sqrt{RRC}$, and NR by LNR = $\log_{10}$NR (SNEDECOR and COCHRAN, 1980).

The different analyses of variance for the 3 criteria considered were carried out according to the following general model:

$$Y_{ijkl} = \mu + E_i + B_{ij} + C_k + A_l + (EC)_{ik} + (EA)_{il} + (CA)_{kl} + \varepsilon_{ijkl}$$

where:

Y_{ijkl}= value of the plot submitted to the i^{th} level of factor *(sub)* experiment, j^{th} level of factor *block,* k^{th} level of factor *cutting category* and l^{th} level of factor *auxin;*

μ = overall mean of the trial;

E_i = effect of the factor *sub-experiment,* $1 \leq i \leq 2$;

B_{ij} = effect of the factor *block j within sub-experiment i,* $1 \leq j \leq 3$;

C_k = effect of the factor *cutting category,* $1 \leq k \leq 6$;

A_l = effect of the factor *auxin,* $1 \leq l \leq 2$;

$(EC)_{ik}$ = effect of the interaction of *sub-experiment* and *cutting category;*

$(EA)_{il}$ = effect of the interaction of *sub-experiment* and *auxin;*

$(CA)_{kl}$ = effect of the interaction of *cutting category* and *auxin;*

ε_{ijkl} = residual error.

Due to imbalance in the data between "exp.1" and "exp.2", cutting category *D* was discarded from the analyses of variance combining these 2 sub-experiments.

Treatment means were compared using the STUDENT-NEWMAN and KEULS test when in F-tests the null hypothesis was rejected ($P \leq 0.05$).

PEARSON's correlation coefficients between the 3 variables, RRC being replaced by ASRRC and NR by LNR, were calculated computing N = 47 elementary plot mean values with high enough rooting rates corresponding to cutting origins *B1, B2, B3* and *C,* treated and not treated with auxin, exp.1 and 2 combined, with at least 1 rooted cutting per elementary plot.

Results

General outlines

Mean values for the various criteria assessed corresponding to the different treatments are given in *table 1.* Similar overall rooting rates were obtained for exp.1 and exp.2 at the end of the 2 month rooting period, 58.0% (174/300) and 56.7% (204/360) respectively, notwithstanding the noticeable score variations between the 2 sub-experiments, and especially in terms of reactivity to exogenous auxin. Only very few cuttings remained alive without having formed roots (7 out of 300 for exp.1 and 12 out of 360 for exp.2). For both sub-experiments, cuttings with phyllodes originating from decapitated stumps *(B3)* rooted best, with overall rooting rates of 88.3% for exp.1 and 81.7% for exp.2. The 2 other criteria, namely number of roots and root length, showed differences in plant material responsiveness with higher overall scores for exp.1.

Correlations

Correlation coefficients between the 3 characters over both experiments were all highly significant establishing that the

Table 1. – Mean values, for "exp. 1", "exp. 2" and the combination ("Combi."), of the 3 criteria assessed – RRC: rate of rooted cuttings; NR: number of roots per rooted cutting; Lenght: length of the longest root per rooted cutting –, in relation to the experimental factors investigated. Means followed by letters only were submitted to the analysis of variance and compared (STUDENT-NEWMAN and KEULS test). Within each column, letters distinguish means which are significantly different at the 5% level.

CRITERIA	RRC (%)			No of plots	NR			Length (cm)			No of plots
FACTORS	Exp.1	Exp.2	Combi.		Exp.1	Exp.2	Combi.	Exp.1	Exp.2	Combi.	
CUTTING CATEGORY											
A	8.3*c*	1.7*c*	5.0*d*	6+6	2.7	1.0*	2.3	8.7	6.0*	8.0	3+1
B1	46.7*b*	76.7*a*	61.7*bc*	6+6	3.7*a*	5.8*a*	4.7*a*	8.7*b*	9.9*a*	9.3*a*	6+6
B2	86.7*a*	63.3*a*	75.0*ab*	6+6	4.7*a*	3.5*b*	4.1*a*	12.2*a*	8.4*b*	10.3*a*	6+6
B3	88.3*a*	81.7*a*	85.0*a*	6+6	5.3*a*	2.6*bc*	4.0*a*	12.4*a*	7.1*bc*	9.8*a*	6+6
C	60.0*b*	38.3*b*	49.2*c*	6+6	4.6*a*	2.7*c*	3.7*a*	9.1*b*	6.0*c*	7.7*b*	6+5
D		78.3*a*	78.3	6		2.5*c*	2.5		5.3*c*	5.3	6
AUXIN											
Control	46.7*b*	64.4*a*	56.4*a*	15+18	2.7*b*	2.1*b*	2.3*b*	9.9*b*	7.9*a*	8.8*a*	13+16
Seradix 3	69.3*a*	48.9*b*	58.2*a*	15+18	5.9*a*	4.8*a*	5.4*a*	10.8*a*	6.7*b*	8.8*a*	14+14
OVERALL SCORES	58.0	56.7	57.3	30+36	4.4	3.4	3.8	10.4	7.4	8.8	27+30

*) Based on one observation since only one cutting not treated with auxin rooted.

higher the rooting rate, the more adventitious roots formed per rooted cutting and the longer these roots.

Analyses of variance and comparison of means

The general analyses of variance of data the mean values of which are reported in *table 1* established that:

1. The various categories of cuttings tested influenced markedly ($P < 0.0001$) the overall rooting rates and length of the newly formed roots. The analyses of variance carried out on the mature-like cuttings only (*Table 2*) demonstrated marked differences in terms of rooting rates and root length ($P < 0.0001$) depending on plant origin, whereas only rooting rate scores were found to be significantly ($0.01 < P < 0.05$, *Table 3*) influenced by the different morphology of the compared cutting classes (*B1, B2, B3*). In both cases, the STUDENT-NEWMAN and KEULS test established that the mature like cuttings produced by the stump (*B3*) rooted in greater amount than the other cutting categories. The interactions reported in *table 2* and *3* between the subexperiments and the different factors investigated are consistent with the score variations from one experiment to the other reported in *table 1*.

Table 2. – Analyses of variance for the rooting rate (expressed by ASRRC), the number of roots (expressed by LNR) and the lenght of the longest root per rooted cutting for the different origins of mature-like cuttings only, "exp. 1" and "exp. 2" combined (see text for more information).

		ASRRC			LNR		Length	
Source	DF[1]	MS[2]	F[3]	DF	MS	F	MS	F
Exp. (E)	1	0.188	4.5*	1	0.321	26.2***	96.625	73.0***
Block	4	0.041	1.0	4	0.007	0.6	0.856	0.6
Origin (O)	2	2.790	66.1***	1	0.014	1.2	26.709	20.2***
Auxin (A)	1	0.039	0.9	1	0.790	64.5***	0.444	0.3
E x O	2	0.028	0.7	1	0.002	0.2	6.227	4.7
E x A	1	0.632	15.0***	1	0.045	3.7	9.336	7.1*
O x A	2	0.076	1.8	1	0.081	6.6*	0.132	0.1
Error	22	0.042		12	0.012		1.324	

[1]) Degrees of freedom.
[2]) Mean square.
[3]) Value of FISCHER'S statistical test with significance levels:
*) $p \leq 0.05$; **) $p \leq 0.01$; ***) $p \leq 0.001$.

Table 3. – Analyses of variance for the rooting rate (expressed by ASRRC), the number of roots and the lenght of the longest root per rooted cutting for the different morphological types of stump cuttings only, "exp. 1" and "exp. 2" combined (see text and *table 2* for more information).

		ASRRC			LNR		Length	
Source	DF	MS	F	DF	MS	F	MS	F
Exp. (E)	1	0.002	0.0	1	0.045	2.8	60.140	29.6***
Block	4	0.046	1.0	4	0.031	1.9	2.393	1.2
Morphology (M)	2	0.236	5.2*	2	0.021	1.3	3.281	1.6
Auxin (A)	1	0.001	0.0	1	0.702	43.2***	0.000	0.0
E x M	2	0.314	7.0**	2	0.125	7.7**	34.997	17.2***
E x A	1	0.434	9.6**	1	0.014	0.9	23.994	11.8**
M x A	2	0.011	0.2	2	0.002	0.1	0.034	0.0
Error	22	0.045		22	0.016		2.031	

2. Auxin treatment with Seradix 3 had no overall significant effect on rooting rate nor on root length, but it resulted in a remarkable increase ($P < 0.0001$) of the number of adventitious roots formed (see *Table 1* for accurate data). The pronounced "experiment x auxin" interactions pointed out in *tables 2* and *3* were caused by differences in response to Seradix 3 treatment for rooting rates and root length. It was promotive in exp.1 and inhibitive in exp.2.

Discussion

The inability of *Acacia mangium* cuttings to survive after 2 months in rooting conditions in the absence of adventitious roots has already been observed (POUPARD *et al.*, 1994) and can be associated with the very limited potential for callus formation in this species in contrast to others like *Sequoia sempervirens* (MONTEUUIS *et al.*, 1987) or *Tectona grandis* able to remain alive several months in similar rooting conditions providing a big basal callus has formed.

A previous investigation (POUPARD *et al.*, 1994) established the advantage of using terminal shoot cuttings as compared to nodal cuttings which were found less responsive to adventitious rooting. The present experiment shows however that the potential to form adventitious roots of such a plant material can vary greatly according to the age of the donor plant this type of cuttings were removed from, although all of them displayed similar mature-like morphological foliage features. The fact that cuttings coming from the crown of 4-year-old ortets, the oldest position from the ontogenetical ageing standpoint (FORTANIER and JONKERS, 1976), demonstrated the lowest very limited capacity for rooting is consistent with many observations (MONTEUUIS, 1985; BON *et al.*, 1994). More surprising is the remarkable rootability of shoots sprouting from stumps, chronologically as old as the ortets, which rooted better than their homologs from same age stock-plants, probably inappropriately managed in terms of feeding and hedging operations. The scores obtained for these terminal shoot cuttings issued from stump sprouts were higher than those observed in similar conditions by POUPARD *et al.* (1994), but the top cuttings in this latter case originated from longer sprouting shoots which were therefore ontogenetically older than the ones used in the present experiment. The same argumentation can be applied to terminal cuttings removed from the 1-year-old seedlings for which greater rooting potential than those observed for the sprouts could have been expected in the case of ontogenetically younger top cuttings produced by less-aged seedlings (DARUS, 1991).

By contrast with other species (MONTEUUIS, 1985), foliar, features as indicators of phase change do not appear to be reliable markers of rooting potential in *Acacia mangium* since for the stump sprout origin, mature-like cuttings rooted better than the juvenile-like ones. In fact, the juvenile phase corresponding to the production of the composed leaves is very short-lived, limited in seedlings to a few weeks during which the maturation process seems to progress quickly as reflected by the noticeable morphological changes from one leaf to the next. Flowering stage is attained around 3- to 4-years on seedlings for this short-lived species. This developmental pattern is even more time-restricted in the case of shoots sprouting from mature stumps. The juvenile stage as reflected by leaf morphology seems to be ephemeral, evolving a lot within a few days. This could account for the rootability variations noted between shoots with composed and intermediate leaves. Another argument to consider is the extreme tenderness of these two categories of cuttings which increase the risks of irreparable stress affecting the rooting potential of these

shoots, whereas the number of roots and the root length scores refute any deficiency in endogenous energy in comparison with other categories.

Contrary to the previous experiment (POUPARD *et al.*, 1994), treating the base of cuttings with "Seradix 3" had, overall, no effect on the rooting rates, with noticeable differences in terms of plant material responsiveness to this exogenous auxin between the 2 sub-experiments. Such variations of rootability in relation to auxin application have already been reported for several temperate arborescent species (MONTEUUIS and PAGES, 1987; MONTEUUIS *et al.*, 1987) arguing about physiological changes in plant material connected with the seasons. Although not exposed to such seasonal contrasts as in temperate countries, the physiological status of the *Acacia mangium* plant material investigated is susceptible to differences from one sub-experiment to the other one according to fluctuations of environmental conditions such as natural photoperiod, rainfall and possible interference of endogenous rhythms the existence of which has already been established for many tropical tree species (HALLE *et al.*, 1978). Such modifications of the physiological status of plant material in time could account for the numerous interactions pointed out between investigated factors and sub-experiments.

Conclusion

The observations emerging from this experiment tend to establish the preponderance of physiological condition of the plant material collected over chronological and even ontogenetical ageing considerations when aiming to get cuttings rooted, as has already been argued (BORCHERT, 1976; MONTEUUIS, 1989). In particular it appears that mature *Acacia mangium* can be cloned with great success from stump produced mature-like cuttings, providing these latter have been collected at the suitable stage of development. Without underestimating the real risks that stumps may not sprout once the selected trees have been felled and the lack of information regarding further behavior of these cuttings, such a potential for adventitious rooting from mature genotypes deserves special consideration for tree improvement and development programmes of *Acacia mangium*.

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In Vivo Grafting and *In Vitro* Micrografting of *Acacia mangium*: Impact of Ortet Age

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Abstract

The possibilities of vegetatively propagating juvenile – 6-month-old – and mature – 3 to 5 year-old – *Acacia mangium* ortets by grafting were investigated using *in vivo* and *in vitro* techniques. The average success rates obtained for *in vivo* top-cleft grafting were 49% for scions coming from juvenile plant material and 0% when collected from mature ortets. *In vitro* micrografted apices gave rise to 52% and 46% of successfully established micrografts for the juvenile and the mature plant material respectively. No significant difference between juvenile and mature origins in terms of grafting success was observed for *in vitro* micrografting of shoot apices. However, the ones coming from the juvenile ortets elongated more readily than those from the mature origin which were more prone to rest. Overall, the *in vitro* micrografting technique used appeared to be an helpful tool for vegetative non-destructive propagation of mature selected *Acacia mangium* ortets, apparently recalcitrant to more conventional *in vivo* grafting techniques.

These results are discussed in terms of scion size and the related potential for grafting in relation to the age of the ortet.

Key words: Acacia mangium, age, grafting, *in vitro,* micrografting, ortet, shoot apex, vegetative propagation.

FDC: 165.442; 176.1 *Acacia mangium.*

Introduction

Grafting has been extensively used for centuries for asexually propagating tree species, mainly for fruit production. This vegetative propagation technique is still broadly utilized in

horticulture (HARTMANN et al., 1990) but also for mass clonal propagation of rubber trees. In forestry, grafting has been mainly used for seed orchard establishment or as a source of vegetative propagules for clonal forestry (ZOBEL and TALBERT, 1984).

Micrografting has been developed more recently (BURGER, 1985; JONARD, 1986) and consists of grafting in aseptic conditions of a miniaturized scion onto an *in vitro* grown rootstock. The resulting *in vitro* micrograft and the plant material deriving from it can be further cultivated in tissue culture conditions, or acclimatized to outdoors. In addition to the benefits of traditional grafting, micrografting tiny shoot tips can be an efficient means of regenerating plant material free of endogenous contaminants (JONARD, 1986; HARTMANN et al., 1990) and with enhanced potential for true-to-type cloning from mature plants (FRANCLET, 1983). The possibility to micrograft less differentiated shoot tip tissues may help also in reducing compatibility problems between scion and stock (LACHAUD, 1975; JONARD, 1986).

The benefits of applying grafting and micrografting to *Acacia mangium* are obvious, considering the need to improve the genetic quality of the planting stock of this attractive fast growing species with increasing crop potential under humid tropics, especially in South East Asia. So far, indeed, air layering or "marcottage" (HARTMANN et al., 1990), although not easy to perform and in spite of rather moderate success, has been the most widely used non destructive method to vegetatively multiplying mature *Acacia mangium* trees recalcitrant to propagation by cuttings (POUPARD et al., 1994).

The capacity for grafting and micrografting of juvenile and mature *Acacia mangium* trees was therefore investigated and the results are reported in this paper.

Material and Methods

Scion source

Mature plant material scions were collected from the basal part of the crown of *Acacia mangium* trees, provenance Papua New Guinea, growing outdoors in Sabah (East Malaysia), 3-year-old since seed germination when the first grafting experiments started, and which just entered the flowering stage.

Juvenile plant material scions originated from 6 to 8 month-old seedlings, provenance Papua New Guinea, container-cultivated in the nursery, close to the mature ortets.

Several sample collections were made at different dates simultaneously for the two age classes which were systematically paired during all the grafting and micrografting procedures.

Grafting technique

Preliminary experiments (data not reported) established the superiority of the "top-clef" grafting technique (HARTMANN et al., 1990) over various other types of grafting for *Acacia mangium*, and this was therefore selected. To ensure a proper matching of stem size between scions and rootstocks, mature plant material scions were collected from the basal part of 10 cm to 20 cm long epicormic shoots arising from low branches of the trees, whilst juvenile scions were taken at about two thirds of the total height of the seedlings. Regardless of the age class, scions consisted of 2 node stem portions, 5 cm to 7 cm in length, with about two thirds of the surface of the phyllodes removed in order to lower evapotranspiration and to reduce water stress risks. The longer basal internode, trimmed to form a "V" 15 mm to 25 mm in length, was then inserted into the vertical slit made in the central part of the one third decapitated stem, about 3 mm in diameter, of the rootstock, a 6 to 8 month-old seedling of the same characteristics as the ortets used as source of juvenile plant material scions. Parafilm tape was used to tie the scion to the stock, and the union was covered with grafting wax, following the recommendations for top-cleft grafting (HARTMANN et al., 1990). The grafted stocks were then placed under 50% shade with intermittent-mist water sprays provided by a mist system (POUPARD et al., 1994) during 3 to 4 weeks to avoid any desiccation damage until the union was successfully established.

Micrografting technique

The *in vitro* rootstocks were obtained from *Acacia mangium* seeds, provenance Papua New-Guinea, first soaked for 5 seconds to 10 seconds in boiling water, then surface-sterilized by immersion for 5 min in 70% ethanol and then in 1‰ $HgCl_2$ aqueous solution. After 3 abundant rinses in sterilized ultra-pure water, the seeds were placed individually in aseptic conditions on 20 mm x 30 mm "Sorbarod" cellulosic plugs in 21 mm x 150 mm glass test tubes covered with polypropylene caps. The plugs had been previously saturated with 5 ml of a liquid medium consisting of half-strength MURASHIGE and SKOOG (1962) macro and micronutrients, with 20 gl^{-1} sucrose and with pH adjusted to 5.5 to 5.6 prior to autoclaving at 120 °C and 95 kPa for 20 min. Cultures were maintained under a 16-h photoperiod (50 to 60 µmol m^{-2} s^{-1}, "TLD 36W/84 Philips" fluorescent lamps) at 28/22 ± 2 °C light/dark. In these conditions 60% to 80% of the seeds germinated to develop within 2 to 3 months into young seedlings with elongating epicotyl suitable for grafting.

The scions used for micrografting originated from shoot apical portions of the two categories of plant material. Immediately after collection, the shoot tips were sprayed with 70% ethanol, then wrapped in ultra-pure water moistened tissue paper before dissection under a binocular microscope with a cold light source. From that stage onwards, all the manipulations were performed under a laminar flow hood, in aseptic

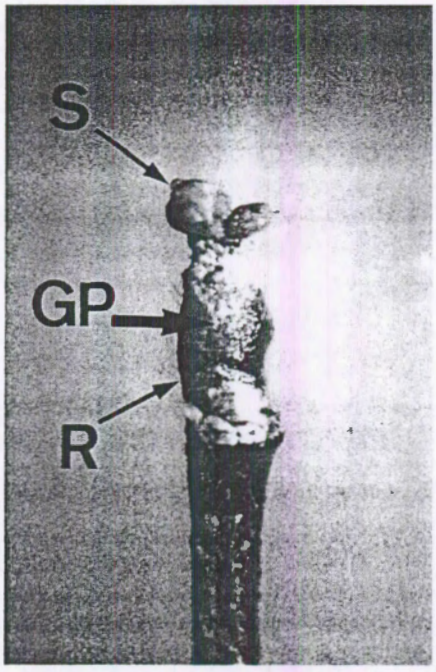

Figure 1. – In vitro micrografted shoot apex (S) of mature *Acacia mangium* ortet starting to elongate, with its "V" shaped basal wedge connected to the rootstock (R) tissues at the grafting point (GP).

conditions. After removal of the outer young phyllodes, the shoot apex, ranging from 300 µm to 400 µm in height, plus a short "V" shaped basal wedge of underlying tissues, was excised to be quickly and carefully inserted into a small vertical slit made in the center of the top cut surface resulting from the decapitation of the rootstock epicotyl, like for a miniaturized "cleft-graft" (HARTMANN et al., 1990), as illustrated in *figure 1*. The possibility to draw the rootstock out of the tube without damaging its root system was achieved thanks to the "Sorbarod" plug which facilitated the manipulations. Once grafted, the seedlings were returned to the initial environmental conditions taking care to remove the numerous axillary shoots produced by the stock to avoid competition with scion development.

Evaluation of grafting and micrografting success

Grafting success rate was established 2 months after grafting by recording the number of scions still alive out of the 15 grafts carried out per sampling date for the 2 categories of plant material.

Success rate for micrografting was defined as the number of grafts successfully established (scions still alive and eventually exhibiting some potential for further elongation) out of the 12 micrografts performed for each sample, 3 months after the date of micrografting. In addition, elongating scions were recorded and measured.

The data were statistically analyzed using the χ^2-PEARSON's test or the analysis of variance test (F-test) after the success rates had been transformed to arcsin $\sqrt{x}$ (SNEDECOR and COCHRAN, 1957), to determine significant differences ($P < 0.05$ level of probability) among the 2 origins.

Results

Data provided in *table 1* establish that *in vivo* grafting of *Acacia mangium* was greatly influenced by the age of the ortet ($P < 0.001$, F-test). On average 48.6% of scions coming from juvenile plant material were successfully grafted and developed into new shoots, whereas when using scions from mature ortets all the grafts failed. The marked difference between juvenile and mature plant material as regards their capacity for *in vivo* grafting, disappeared when using the shoot apex micrografting technique. It gave similar average success scores of 51.8% and 45.6% respectively. However, in contrast to *in vivo* grafting several of the successfully established *in vitro* micrografts were still resting 3 months after micrografting. The proportion was significantly higher for micrografts derived from mature ortets ($P < 0.001$, χ^2-test). Only 15 out of 36 had begun to resume growth at that time (*Figure 1*), as compared to 36 out of 42 for the juvenile plant material. Although the growing scions from the juvenile source were generally observed to elongate faster than their homologs from the mature ortets (*Figure 2*), the within sample variability did not allow to point out any significant difference in terms of scion length between the 2 age classes 3 months after micrografting.

Table 1. – In vivo grafting and *in vitro* micrografting success rates for scions collected at different dates from juvenile and mature *Acacia mangium* ortets and submitted exactly to the same experimental conditions.

IN VIVO GRAFTING			IN VITRO MICROGRAFTING		
Dates of scion collection	Success rate		Dates of scion collection	Success rate	
	Juvenile	Mature		Juvenile	Mature
4-12-1991	6/15	0/15	22-9-1994	2/12*	6/12
13-12-1991	5/15	0/15	5-10-1994	5/10	4/11
27-12-1991	2/15	0/15	2-11-1994	6/12	3/10
28-1-1992	12/15	0/15	24-11-1994	7/12	7/12
18-2-1992	8/15	0/15	16-12-1994	7/12	7/11
6-3-1992	11/15	0/15	21-12-1994	5/11	4/11
30-12-1993	7/15	0/15	29-12-1994	10/12	5/12
Average rate of success	51/105	0/105		42/81	36/79
% ± S.D.	48.6±4.9	0		51.8±5.5	45.6±5.6

*) initially 12 micrografts were performed for both ages of plant materials; some were lost due to fungal contaminations.

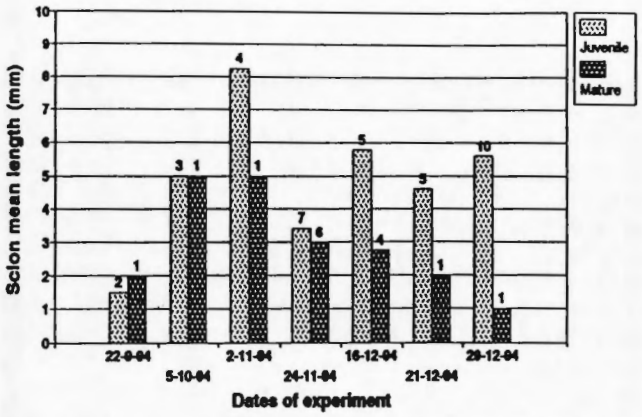

Figure 2. – Comparative mean length 3 months after micrografting of *in vitro* elongating scions collected from juvenile and mature *Acacia mangium* ortets at different dates and submitted exactly to the same experimental conditions. The number of scions which were elongating and measured is given for each sample.

Discussion

The current study indicates that although *Acacia mangium* can be grafted, success rates in *in vivo* conditions are rather low compared to other species and highly dependent upon the age of the donor tree. As a matter of fact, scion capacity to produce a callus, which can be considered as a characteristic wound response in plants (MOORE, 1981) and commonly reported as a prerequisite of grafting success (BARNETT and WEATHERHEAD. 1988; HARTMANN et al., 1990), was observed to be quite weak in this species, especially for mature plant material as was already noticed when studying rooting of cuttings (POUPARD et al., 1994; MONTEUUIS et al., 1995). The fact that flushing shoots produced by regularly hedged 3-year-old stock plants give rise to similar grafting scores as shoots from young seedlings (unpublished data) contrasts with scions taken from crown branches which tends to demonstrate the negative influence of increasing ontogenetical ageing (FORTANIER and JONKERS, 1976) of scions on their ability to be successfully grafted, in the same way as it has been observed for adventitious rooting potential of cuttings (MONTEUUIS et al., 1995).

The micrografting approach seems to neutralize the impact of ontogenetical ageing considered for the whole tree, since the 2 compared origins of micrografted shoot apices did not differ significantly in terms of potential for callus formation, giving rise to similar overall grafting success rates, although the apices originated from mature ortet crown were ontogenetically

older than the ones coming from juvenile seedlings. Particularly, in contrast with the 5 cm to 7 cm long scions used for *in vivo* grafting which all failed, 46% of the mature derived shoot apices produced a callus that allowed them at least to survive. This illustrates the possibility of finding within ontogenetically mature shoots that had already entered the flowering stage some less differentiated shoot tip tissues close to the apical meristem that could express a similar potential, at least for callus formation, as those produced by juvenile seedlings. This has been observed for other species also (MONTEUUIS, 1991). As far as the potential for grafting is concerned LACHAUD (1973) distinguished between ageing of scion tissues, referred to as "âge local" and ageing of the whole donor tree – "âge général". However, the fact that the successfully established micrografted apices of mature origin were more recalcitrant to elongate than their homologs originating from juvenile seedlings might be due to physiological and biochemical differences between the two origins of shoot apices, most probably caused by physiological ageing (LACHAUD, 1975; HACKETT, 1983; MONTEUUIS, 1989). With reference to other works (MONTEUUIS, 1987 and 1991), the possibility to reduce the size of the micrografted shoot apices to only the true apical meristem may help to counteract such negative effects of physiological ageing on the potential for organogenesis of the mature plant material. Experiments currently under way, although technically handicapped by the very tiny size of the *Acacia mangium* shoot apical meristem, may answer to what extent this will prove possible.

Conclusion

Shoot apex micrografting has to be considered as an helpful alternative to *in vivo* grafting for *Acacia mangium*, especially for plant material collected from the crown of mature ortets.

In addition to rejuvenation prospects, the importance of which has to be emphasized for clonally propagating true-to-type mature *Acacia mangium* genotypes, micrografting was found to be an efficient technique overcoming tissue culture contamination problems, particularly those of endogenous origin, which are to date serious impediments to micropropagation programmes of this economically important forest tree species.

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Research note

Influence of the grafting technique on meristem micrografting of Douglas-fir

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Key words: *Pseudotsuga menziesii*, cloning, *in vitro*, shoot tips, vegetative propagation

Application. The described technique allows micrografting of shoot meristems of Douglas fir (*Pseudotsuga menziesii*). This should aid vegetative propagation efforts for the species as meristem micrografting can reduce the risk of graft incompatibility and may induce rejuvenation of mature selected genotypes.

Abstract. Three techniques for *in vitro* micrografting of shoot meristems from a 15-year-old clone of Douglas fir (*Pseudotsuga menziesii*) were compared. Although micrografting was feasible with all three procedures, the success rate was greatly influenced by the type of graft used. The best score (50% success) was obtained when using a miniaturized side-grafting technique. The micrografts, which could be easily acclimatized to *in vivo* conditions, exhibited however substantial variability with regard to scion development.

Introduction

Douglas fir (*Pseudotsuga menziesii*) is one of the world's most important timber species and has been introduced into many temperate countries from its native range of western North America. Given its attractive features, many years of effort have been devoted in tree improvement programs toward development of vegetative propagation techniques. However, progress has been handicapped by reactions of incompatibility in grafts, and by the negative effects of the maturation process on the adventitious rooting capacity of the cuttings (Goldfarb and Zaerr 1989).

Micrografting apical meristems of mature selected Douglas fir genotypes should constitute a judicious means to solve these problems as recently noted by Pullman and Timmis (1992), and combine the advantages of meristem cultures with those of grafting as broadly reviewed by Burger (1985) and Jonard (1986). The possibility of recovering juvenility (Doorenbos 1953; Tranvan et al. 1991; Pullman and Timmis 1992), especially true-to-type cloning ability

for grafted plant material (Franclet 1983; Pliego-Alfaro and Murashige 1987; Huang et al. 1992) and the expectation of reducing incompatibility risks by grafting meristematic tissues are major arguments in favor of this technology, which has already been successfully applied to other coniferous species (Monteuuis 1986; Dumas et al. 1989; Goldfarb et al. 1993).

Materials and methods

Obtaining in vitro rootstocks

The *in vitro* seedlings used as rootstocks were obtained from Douglas fir seeds that were surface-sterilized by immersion for 20 min in aqueous 7% $Ca(OCl)_2$, followed by 3 min in 70% ethanol. Seeds were then rinsed 3 times and retained overnight in sterile distilled water. Individual seeds were placed on 20 × 30 mm cellulose acetate "Sorbarod" cylindric containers (Baumgartner Papier SA, Lausanne, Switzerland) in 25 × 200 mm glass test tubes. The Sorbarods were previously saturated with 5 ml of liquid medium consisting of Lepoivre's (Quoirin et al. 1977) half strength macronutrients, Murashige and Skoog (1962) micronutrients, 30 g/l sucrose and 5 g/l activated charcoal. The medium was autoclaved at 120 °C for 20 min.

Cultures were maintained under a 16 h photoperiod with a photon flux density of 60–70 μmol m^{-2} s^{-1} provided by "Sylvania Cool White" fluorescent lamps at 25/22 ± 2 °C, light/dark. In these conditions, 50 to 70% of the germinants developed, within 2 to 3 months, into young seedlings with fully expanded cotyledons and an elongating epicotyl suitable for grafting.

Scion origin

The apical meristems used as scions originated from vegetative buds produced by rooted cuttings obtained by serial propagation of a single genotype of Douglas-Fir (*Pseudotsuga menziesii*) aged 15-years since seed germination. These 3 to 4-year-old rooted cuttings were kept intensively cultivated and hedged outdoors at AFOCEL, Domaine de l'Etançon, France. Several sample collections corresponding to different dates were performed during the year.

Grafting technique

Immediately upon collection, buds with a short portion of shoot underneath were dipped for a few seconds in 70% ethanol. The apical meristem was aseptically dissected from each bud with the aid of a binocular microscope

Fig. 1. Micrografted meristems of *Pseudotsuga menziesii* (arrowheads) illustrating the "side-grafting" (a), the "cleft-grafting" (b) and the "top-grafting" (c) techniques described in the text.

and a cold light source. Apical meristems ranged from 100 to 250 μm in height and from 200 to 400 μm in width according to the seasonal growth phases (Owens and Molder 1973).

Three different techniques of micrografting (*i* "side-grafting", *ii* "cleft-grafting" and *iii* "top-grafting") involving at least 10 meristems per treatment were compared for each collection. "Side-grafting", originally developed on *Sequoiadendron giganteum* (Monteuuis 1986) consisted of inserting the excised meristem with a short wedge of underlying tissues into a lateral cut made between two cotyledons of the *in vitro* seedling rootstock (Fig. 1a). In "cleft-grafting" the meristem was also removed with a short "V" shaped basal wedge of underlying tissues to be inserted into a small vertical split made in the center of the top cut surface, resulting from the decapitation of the elongating epicotyl of the rootstock (Fig. 1b). "Top-grafting" as initially described by Navarro et al. (1975) consisted of placing the horizontal cut section of the excised meristem onto the top cut surface of the decapitated rootstock epicotyl (Fig. 1c). The basal wedges produced in techniques *i* and *ii* did not exceed 250 μm in height. Once grafted, specimens were returned to the environmental conditions as previously described.

Care of the grafted plants and acclimatization

Every 2 to 3 months, 2 ml of the sterilized liquid medium were provided in aseptic conditions to the grafted rootstocks maintained in their original test tube. After the grafted meristem underwent new organogenesis (leaf formation and further expansion) indicating that connection with the stock had occurred (Fig. 2), the rootstock epicotyl ("side-grafting" technique) was cut off. Similarly, any axillary shoots produced by the stock was systematically removed to avoid competition.

Fig. 2. First phases of scion development showing graft union callus, expansion of new leaves, and stem elongation.

The transfer of the grafted plants to the greenhouse was achieved preferably in spring. Plants were carefully removed by hand from the Sorbarods, potted in 1-L containers filled with horticultural peat-perlite (50:50, v/v) mixture and watered with an aqueous solution of Aliette (2 g/l). The pots with the grafted plants, sprayed with 2 g/l of Benlate, were individually covered with thin polyethylene bags to maintain high relative humidity (80–85%). After 2 to 4 weeks, the bags were progressively removed to acclimatize the specimens (Fig. 3), and their development was then stimulated by application of N-P-K liquid fertilizers.

Results and discussion

The rate of successfully established micrografts (organogenic grafted meristems divided by the total number of grafts performed) was significantly influenced ($P < 0.001$ when applying the Chi-square test) by the grafting technique. On average, the side-grafting technique (*i*) gave the best results with 50% successful grafts (41 out of 82), whereas only 28.4% (23/81) and 8.6% (7/81) of the cleft-grafted (*ii*) and top-grafted (*iii*) meristems respectively responded positively. For meristems removed with a basal wedge of tissues, grafting between two cotyledons on the side (*i*) was found more efficient than on the top (*ii*) of the seeding stock (50% vs 28.4%, $P < 0.05$

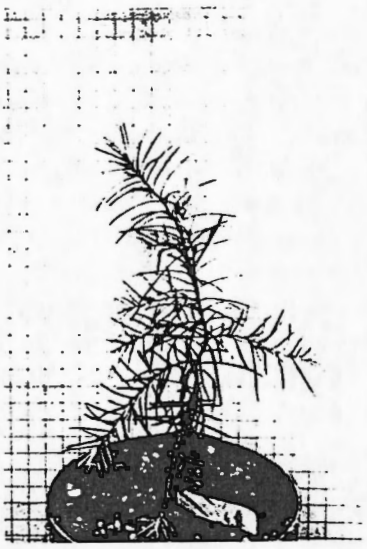

Fig. 3. Acclimated *Pseudotsuga menziesii* micrograft. The sides of the background squares are each 1 cm.

– Chi-square test). For the grafts performed on the top of the decapitated rootstocks, it appeared that meristems removed with a wedge of underlying tissues (*ii*) reacted significantly better than those without (*iii*) (28.4% vs 8.6% respectively, $P < 0.01$ – Chi-square test).

As far as we are aware, this is the first work to report micrografting of Douglas fir shoot meristems with success rates that could make the technique a viable substitute for meristem culture on synthetic media, which itself remains problematic (Goldfarb and Zaerr 1989, Pullman and Timmis 1992). It should be emphasized that the techniques developed, especially the side-grafting, required high dexterity and rapidity. In this respect, the "Sorbarod" plug greatly aided the removal of specimens from their culture tubes without damaging the root system. The "Sorbarod" also facilitated the transfer to horticultural substrate for this species which is recognized as having a sensitive root system. Using meristems instead of buds for *in vitro* grafting of outdoor grown Douglas fir plant material allows one to minimize sterilization techniques that can be damaging for the grafted tissues, and dodges the problem of stem size differences between scions and *in vitro* produced stocks noticed by Pullman and Timmis (1992). From the data communicated, it appears that *in vitro* grafting of meristems could give better results than grafting of buds removed from similar age plants. This could be due to differences in terms of tissue maturity (Lachaud 1975) between the grafted buds and the juvenile

in vitro rootstock which are not as pronounced with meristems and the same rootstock.

It has to be observed, however, that whatever the technique used, the successful micrografts exhibited noticeable variability in terms of further development of the scion, from a resting scaly bud to an actively expanding juvenile-like shoot (Fig. 3). Such intraclonal variability was observed even for meristems removed at the same date from the donor plant. This could be due to severals factors such as the quality of the graft connection, the genetic diversity of the seedlings used as rootstocks, or the physiological status of the excised meristem at the time of its removal from the donor plant. Further studies on heterogeneity of responses observed here, as well as for other species (Dumas et al. 1989, Tranvan et al. 1991), are needed to draw the best benefit of this technology.

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Survival and growth of eastern white pine shoot apical meristems *in vitro*

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Abstract

Shoot apical meristems of seedling and mature eastern white pine trees were excised and grown *in vitro*. Placing the meristems on filters instead of directly on agarose-solidified nutrient medium enhanced survival of both juvenile and mature meristems. Applying forcing treatments to mature branches improved survival and growth of dissected meristems compared with meristems from non-forced branches in experiments conducted over two years. No consistent differences were observed among 2-, 4-, and 6-week forcing treatments. Including 5.37 nM (0.001 mg l^{-1}) 1-naphthaleneacetic acid in the culture medium did not affect meristem survival or growth. Some meristems from seedlings grew rapidly, produced primary leaves, underwent internode elongation, and in three cases, produced adventitious roots. Meristems from mature trees did not grow as rapidly as seedling meristems. The leaves produced by mature meristems appeared to be scale leaves and a few of these had brachyblast primordia in the axils. The shoots derived from mature meristems did not produce adventitious roots.

Abbreviations: BA – 6-benzyladenine; NAA – 1-naphthaleneacetic acid

Introduction

The shoot apical meristem gives rise to all aerial plant parts (reviewed in Sussex, 1989; Medford, 1992). In perennial plants, the developmental characteristics of shoots can vary according to a process known as maturation (reviewed in Greenwood and Hutchison, 1993; Lawson and Poethig, 1995). Leaf morphology is one characteristic that changes with plant age. Another important maturational change is the decline in the ability of stem cuttings to form adventitious roots as the donor plant matures (reviewed in Hackett, 1988). It has been hypothesized that the maturational state of a tissue is determined by the meristem from which it was derived (Bonga, 1987). The maturation state of a meristem, in turn, may be affected by the signals received as a result of its location on the plant. It has been observed that removing meristems from the mature surrounding tissues of older plants can release the meristems juvenile potential (Monteuuis, 1988). For example, an excised shoot apical meristem of a 100-year-old *Sequoiadendron giganteum* tree yielded shoots that displayed juvenile organogenic, morphological, (Monteuuis, 1991) and biochemical (Bon and Monteuuis, 1991) characteristics.

Because of the importance of the shoot apical meristem for woody plant development, its potential importance in determining maturation, and the difficulty of asexually propagating mature eastern white pine (*Pinus strobus* L.), it would be advantageous to have a system for culturing shoot apical meristems of this species *in vitro*. This system could potentially be used to study the internal and external factors controlling development and for testing for rejuvenation by meristem excision and culture.

In preliminary experiments, cultured meristems from both mature trees and seedlings showed very poor survival. We tested a number of medium components including sugar type and concentration, inorganic nutrient formulation, gelling agent, auxins and

cytokinins; but none of these factors substantially improved survival. As a result of these preliminary experiments, we chose to investigate whether culturing the meristems on a filter placed upon the agarose-solidified medium improved survival. Obtaining higher survival would allow us to test the effects of forcing treatments on meristem survival and growth and for possible rejuvenation of excised meristems.

The objectives of these studies were to determine if:

- meristem survival is improved by culturing meristems on filters,
- meristem survival and growth is affected by applying forcing treatments to the donor branches, and
- removing mature meristems from surrounding tissues results in rejuvenation in eastern white pine meristems.

Materials and methods

Filter experiment

Mature meristems were obtained from branches in the upper half of the crowns of four ramets of one eastern white pine clone (H-130). The ramets had been grafted into the U.S. Forest Service, Oconto River Seed Orchard near White Lake, Wisconsin. H-130 was originally selected in 1966 and was estimated to be 50 years from seed at that time. Thus, by the time we first collected branches from the ramets (in 1992), the material was approximately 76 years from seed. Branches up to 2 m long were collected, cut into sections no longer than 1 m, and placed in plastic bags with moist paper towels. Branches were collected on January 6, 1992 and stored for six months at -20 °C. Two weeks before dissection, branches were transferred to 4 °C. Terminal meristems of primary, secondary and tertiary branches were excised and cultured.

Meristems from young seedlings were excised and cultured for comparison with the mature meristems. Open-pollinated seeds from another tree were stratified at 4 °C for 6 to 8 weeks. Seeds were sown in plastic tubes ("Pine Cells," Stuewe & Sons, Inc., Corvallis, OR) containing a mix of composted pine bark, peat, vermiculite, and perlite ("Universal Mix," Strong Lite Horticultural Products, Seneca, IL) and placed in a greenhouse at a temperature of 23-26 °C. Ambient light was supplemented with high-pressure sodium lamps (Sylvannia LU400) at a photosynthetic flux of 100 μmol m^{-2} s^{-1} to provide a 16-h photoperiod. Meristems were excised from the seedlings six weeks after sowing.

Surface disinfestation procedures were not used on buds, shoots or seedlings before meristem dissection. Dissecting tools were sterilized in 70% ethanol between the removal of external structures such as needles or bud scales and the actual meristem excision to prevent contamination of the meristems with microorganisms present on the external structures. Excisions were performed in a laminar-flow hood using a dissecting microscope. The meristematic dome plus the 4-7 smallest leaf primordia were separated from the shoot using a cut transverse to the shoot axis. Excisions were made with carbon steel razor blade shards mounted in a blade holder. Excised meristems were placed either directly on nutrient medium (approximately 4.5 ml per dish) or on filters upon the same medium in 35-mm diameter, disposable petri dishes (Falcon 1008, Becton Dickinson Labware, Lincoln Park, New Jersey). The filters were 25 mm in diameter, had a 5-μm pore size, and were made of mixed cellulose esters (MicronSep, Micron Separations Inc., Westboro, Massachusetts). The medium consisted of 1/2-strength Schenk and Hildebrandt (SH) (1972) salts, full-strength SH vitamins and 30 g l^{-1} sucrose. It was adjusted to pH 5.75 prior to adding 5 g l^{-1} agarose (Ultrapure, Life Technologies Inc., Gibco/BRL, Gaithersburg, Maryland) and autoclaving.

At the time of excision, meristems ranged from 0.3 to 0.5 mm in diameter at the base. Juvenile meristems were consistently smaller than mature ones with a diameter of approximately 0.3 mm, whereas mature meristems ranged from 0.4 to 0.5 mm. Plates containing the meristems were placed in a growth chamber at 23±1 °C. Light was provided for 16 h per day from GTE Sylvania GroLux fluorescent tubes at a photosynthetic flux of 40 to 50 μmol m^{-2} s^{-1}. Three weeks after excision, the meristems, or the filters supporting the meristens, were transferred to new plates containing fresh medium of the same composition. After six weeks in culture, the meristems were scored for survival using a dissecting microscope. Meristems were considered alive if they had become green and had exhibited some growth since dissection. Meristems contaminated with microorganisms were considered as missing data.

The experiment was a two by two factorial. One factor was age of the source material for the meristems (seedling or mature tree) and the other factor was the presence or absence of filters between the meristem and the nutrient medium. Thirty meristems were excised

Table 1. Effects of forcing treatments on survival of eastern white pine shoot apical meristems cultured *in vitro* in 1992.

Number of weeks of *in vitro* culture	Number of weeks of forcing treatment			
	0	2	4	6
	Percent survival			
3	53.6[1] b[2]	86.0 a	90.0 a	81.7 a
	(6.7)	(4.6)	(3.9)	(5.0)
6	30.9 b	64.9 a	73.3 a	63.3 a
	(6.3)	(6.4)	(5.8)	(6.3)
9	18.2 b	42.1 a	42.4 a	35.0 ab
	(5.2)	(6.6)	(6.5)	(6.2)
12	16.4 b	36.8 a	33.9 ab	28.3 ab
	(5.0)	(6.4)	(6.2)	(5.9)
15	12.7 b	36.8 a	28.8 ab	23.3 ab
	(4.5)	(6.4)	(5.9)	(5.5)
18	7.3 b	26.3 a	20.3 ab	16.7 ab
	(3.5)	(5.9)	(5.3)	(4.9)
21	3.6 a	15.8 a	8.5 a	11.7 a
	(2.5)	(4.9)	(3.7)	(4.2)

[1]Each value is the mean (standard error of the mean) of three replications (NAA treatments combined).
[2]Values within a row followed by the same letter are not significantly different ($p \leq 0.05$) by Tukey's test for multiple comparisons.

and cultured in each treatment combination and the experiment was conducted once for a total of 120 meristems. Differences in survival between juvenile and mature meristems and between meristems placed on filters or directly on medium were tested for significance using an analysis of proportions for two-way tables and t-tests (Snedecor and Cochran, 1967).

Forcing experiments

The forcing experiments were conducted over two years. In 1992, branches from the mature trees were collected on April 4, 1992 and stored at -2 °C for 6 months. Branches up to 1 m in length were removed from the freezer either 0, 2, 4, or 6 weeks prior to dissection and placed with their bases in water in the greenhouse under the same conditions as those used for seedling growth. Each week, approximately 5 cm was trimmed from the base of each branch.

Excised meristems were placed on filters (as described above) upon nutrient medium supplemented with 5.37 nM (0.001 mg l^{-1}) NAA or on filters upon medium without NAA. All the meristems within a replication were dissected during a two-day period. Meristems were cultured under the conditions described for the filter experiment. Every three weeks, up to 21 weeks after dissection, filters with explants were transferred to fresh plates. At the same time, each meristem was examined under a dissecting microscope, assessed as alive or dead (as above), and the number of green leaves or leaf primordia counted. The number of green leaves and leaf primordia is a conservative estimate of the total number of leaves initiated, because some primordia were obscured from view by surrounding leaves. We used the number of leaves (and leaf primordia) as an indication of the amount of growth and development that took place in the cultured meristems.

The experiment was a two by four factorial. One factor was the presence or absence of NAA in the nutrient medium and the other factor was the four forcing durations (0, 2, 4 and 6 weeks). Ten meristems were excised and cultured for each treatment combination and the entire experiment was replicated three times at 1-week intervals. Over all treatments and replications, 240 meristems were excised and cultured. Analyses of variance, Tukey's test for multiple comparisons (forcing durations), and LSD tests for two-way comparisons (presence or absence of NAA) were used to test for statistically significant differences at $p \leq 0.05$ (SAS Institute. 1988). During analysis, data sets were tested for normal distribution and constant variance. The survival data set for 1992 was transformed by adding 0.5 and taking the square root prior to analysis. The data set for the numbers of leaves produced did not require transformation.

In 1993, branches for two additional replications of the forcing experiment were collected on March 18, 1993 and stored at -2 °C. Three months later, the two replications of the forcing and NAA treatments were conducted one week apart in the same design as the previous experiment. One hundred sixty meristems were excised, cultured and scored as described above. In 1993, the data sets for survival and number of leaves did not require transformation to satisfy the assumptions for analysis of variance.

Results and discussion

Filter experiment

Juvenile meristems were conical in shape and their height was greater than their width, whereas the mature meristems (from nonforced branches) had a flatter surface and their height was not as great as their width.

Placing excised meristems on filters for *in vitro* culture significantly improved survival ($p < 0.001$) for both juvenile and mature meristems. Six weeks after dissection, 96.6% (28 of 29) of juvenile meristems and 17.9% (5 of 28) of mature meristems on filters were alive, whereas without filters, 3.4% (1 of 29) of juvenile meristems and no (0 of 29) mature meristems survived (filter vs. no filter pooled standard error = 4.0). The difference in survival between meristems from seedlings and mature trees was also statistically significant ($p < 0.001$; pooled standard error = 3.0).

The higher survival of meristems cultured on filters may be because these meristems are situated above the liquid film that forms on the surface of solidified nutrient media. Because of their small size, meristems placed directly on medium tend to become covered by the liquid and may experience anaerobic conditions. When they are placed on filters, meristems still have access to nutrients through their bases that are in contact with the liquid through the capillary action of the filters. The beneficial effects of culturing shoot apices with their bases on solid supports and immersed in liquid medium was reported for *Pinus caribaea* (Skidmore et al,, 1988) and apparently this holds true for the smaller meristem explants in our experiment. Romberger et al. (1970) used cellulose polyacetate filters to collect, sort and grow *Picea abies* meristems, but did not report on the importance of the filters for long-term meristem survival and growth. In addition, as pointed out by these same authors, use of filters makes transfer of cultured meristems to fresh plates more rapid and prevents damage caused by handling the meristems with tools.

The differences in survival between juvenile and mature meristems in this experiment may not have been solely related to maturation. Seedling meristems were from actively growing plants whereas mature meristems were from quiescent material that had been stored under cold conditions for six months. Nevertheless, the increased survival obtained by culturing meristems on filters allowed us to investigate the effects of forcing treatments on meristem growth and development in culture.

Forcing experiments

Forcing treatments resulted in shoot phenology that approximated development in the spring. Terminal buds swelled and opened, followed by growth of the previous year's primordia and elongation of the shoot axis. Forcing treatments also resulted in changes in meristem shape. Meristems from forced branches had heights and widths that were approximately equal as opposed to meristems from nonforced branches that had widths greater than their heights.

Table 2. Effects of forcing treatments on the number of leaves and leaf primordia per surviving eastern white pine shoot apical meristem in 1992.

Number of weeks of *in vitro* culture	Number of weeks of forcing treatment			
	0	2	4	6
	Number of leaves			
3	4.2[1] b[2]	5.3 a	5.6 a	5.3 a
	(0.3)	(0.2)	(0.3)	(0.3)
6	5.1 a	5.4 a	5.8 a	5.7 a
	(0.5)	(0.3)	(0.4)	(0.3)
9	6.8 a	7.3 a	7.1 a	7.9 a
	(0.8)	(0.5)	(0.6)	(0.6)
12	6.8 a	7.9 a	8.1 a	9.6 a
	(0.9)	(0.5)	(0.6)	(0.7)
15	7.1 b	8.7 b	11.0 ab	12.1 a
	(1.9)	(0.9)	(0.8)	(0.7)
18	5.7 b	7.9 b	8.1 b	12.3 a
	(2.7)	(1.1)	(0.9)	(1.4)
21	6.5 b	7.1 b	10.2 ab	14.6 a
	(4.5)	(1.8)	(2.2)	(1.7)

[1]Each value is the mean (standard error of the mean) of three replications with 20 meristems per replication at the beginning of the experiment (NAA treatments combined).
[2]Values within a row followed by the same letter are not significantly different ($p \leq 0.05$) by Tukey's test for multiple comparisons.

In 1992, survival of dissected meristems across all treatments declined from 78.1% after 3 weeks in culture to 10.0% at 21 weeks. Survival was greater in the meristems from branches receiving the 2-, 4-, or 6-week forcing treatment than in meristems from nonforced branches after 3 and 6 weeks in culture (Table 1). After 9 through 18 weeks in culture, survival of meristems from 2-week-forced branches was greater than nonforced meristems, but after 21 weeks in culture there were no significant differences in survival among the forcing treatments.

Forcing treatments, in 1992, affected the number of leaves produced per surviving meristem at some measurement times after dissection. After 3 weeks in culture, meristems from all three forcing treatments had significantly more leaves than meristems from nonforced branches (Table 2). After 6, 9 and 12 weeks in culture there were no significant differences among forcing treatments. After 15, 18 and 21 weeks in culture, the meristems from branches forced for 6 weeks

Table 3. Effects of forcing treatments on survival of eastern white pine shoot apical meristems cultured *in vitro* in 1993.

Number of weeks of *in vitro* culture	Number of weeks of forcing treatment			
	0	2	4	6
	Percent survival			
3	71.8[1] a[2]	97.4 a	100.0 a	97.5 a
	(7.3)	(2.6)	(0.0)	(2.5)
6	38.4 b	94.9 a	85.0 a	95.0 a
	(7.9)	(3.6)	(5.7)	(3.5)
9	36.0 b	89.5 a	62.5 ab	95.0 a
	(7.8)	(5.0)	(7.8)	(3.5)
12	33.3 b	86.8 a	60.0 ab	95.0 a
	(7.6)	(5.6)	(7.8)	(3.5)
15	33.3 b	81.6 a	51.3 ab	95.0 a
	(7.6)	(6.4)	(8.1)	(3.5)
18	28.2 c	81.6 ab	48.7 bc	87.5 a
	(7.3)	(6.4)	(8.1)	(5.3)
21	25.6 b	55.2 a	43.6 ab	80.0 a
	(7.1)	(8.2)	(8.0)	(6.4)

[1]Each value is the mean (standard error of the mean) of two replications (NAA treatments combined).
[2]Values within a row followed by the same letter are not significantly different ($p \leq 0.05$) by Tukey's test for multiple comparisons.

Table 4. Effects of forcing treatments on the number of leaves and leaf primordia per surviving eastern white pine shoot apical meristem in 1993.

Number of weeks of *in vitro* culture	Number of weeks of forcing treatment			
	0	2	4	6
	Number of leaves			
3	4.5[1] b[2]	6.2 a	5.1 b	5.4 ab
	(0.3)	(0.3)	(0.3)	(0.2)
6	5.8 b	7.5 a	6.1 b	6.4 b
	(0.4)	(0.2)	(0.3)	(0.2)
9	5.9 b	9.2 a	7.8 a	8.0 a
	(0.6)	(0.3)	(0.5)	(0.3)
12	5.5 b	10.2 a	8.6 a	9.0 a
	(0.5)	(0.4)	(0.8)	(0.5)
15	6.0 b	11.0 a	10.1 a	9.8 a
	(0.6)	(0.4)	(0.7)	(0.6)
18	5.4 b	11.0 a	10.8 a	10.8 a
	(0.9)	(0.5)	(0.8)	(0.6)
21	4.7 b	10.9 a	11.4 a	12.0 a
	(0.8)	(0.9)	(0.9)	(0.6)

[1]Each value is the mean (standard error of the mean) of two replications with 20 meristems per replication at the beginning of the experiment (NAA treatments combined).
[2]Values within a row followed by the same letter are not significantly different ($p \leq 0.05$) by Tukey's test for multiple comparisons.

had significantly more leaves than those from branches forced for either 0 or 2 weeks.

The overall survival rate of the meristems excised in 1992 was low by the end of the 21-week culture period. Only 23 of 231 meristems (9 excluded, because of contamination) remained alive after 21 weeks. This caused a small sample size for testing differences in the numbers of leaves produced and may have contributed to the differing significance assessments at different measurement times (Table 2). Because of these ambiguities, we elected to conduct two additional replications of the experiment in 1993.

Survival in 1993 was substantially higher than in 1992. After 21 weeks, 51.3% of meristems from all treatments were alive as compared with 10.0% from the prior experiment. Survival was higher in meristems from branches that had been forced for 2, 4 or 6 weeks than in meristems from nonforced branches after 6 weeks in culture (Table 3). Survival was also greater in meristems from branches forced for 2 or 6 weeks than in meristems from nonforced branches after 9, 12, 15 and 21 weeks in culture.

The number of leaves produced on meristems was affected by forcing treatments in 1993. After 3 weeks in culture, meristems from branches forced for 2 weeks had significantly more leaves than meristems from nonforced branches or from branches forced for 4 weeks (Table 4). After 6 weeks in culture, meristems from the 2-week forcing treatment had more leaves than meristems from any of the other treatments. After 9 through 21 weeks in culture, all three forcing treatments resulted in greater numbers of leaves than the nonforced treatments and did not differ amongst themselves. The mean of all forced meristems had increased from 5.5 leaves at week 3 to 11.6 leaves at week 21. In contrast, meristems from non-forced branches increased only from 4.5 leaves per meristem at week 3 to a maximum of 6.0 leaves at week 15 and then declined to 4.7 leaves at week 21.

The higher survival of excised meristems in 1993 compared to 1992 may have been related to differences in the handling of the plant material. The branches for 1992 were stored for twice as long as those for 1993. No visible signs of damage were detected, but reduced vigor from prolonged storage may have been a contributing factor for the lower survival in 1992. Additionally, our meristem dissection technique may have improved in the second experiment as a result of experience the previous year. Romberger et al. (1970)

reported on the necessity of rapid dissection to prevent dessication of the excised meristems. The better survival in 1993 gave us adequate sample sizes to rigorously test the trends observed in 1992 with regard to the effects of the forcing treatments.

The forcing treatments resulted, in general, in meristems that survived better and produced more leaves in culture than nonforced meristems. The season of collection has been reported as affecting the developmental potential of shoot tips of *Abies balsamea* (Bonga, 1981) and *Pseudodotsuga menziesii* (Boulay, 1979), and meristems of *Sequioiadendron giganteum* (Monteuuis, 1991). For the forcing experiments, we collected branches in late winter/early spring (March and April). Although classified by Owston (1969) as dormant, eastern white pine shoots at this time (and location) are more aptly characterized as quiescent. They have received sufficient exposure to low-temperatures that, with the onset of favorable conditions, shoot growth will begin. The quiescent status of the branches was confirmed by the rapid shoot elongation that occurred during the greenhouse forcing treatments. We detected no consistent differences among different stages of active meristems as represented by our 2-, 4-, and 6-week forcing treatments. Additional evidence for high survival and good growth of actively growing meristems comes from separate experiments, in which we collected branches on June 10, 1994 and immediately dissected the meristems. This date was estimated to be six weeks after the first signs of bud break according to visual observations in the seed orchard. The survival rates and numbers of leaves initiated for excised meristems from this material was comparable to the meristems from the forced branches described in this paper. Natural bud flush and *in vitro* forcing treatments have also been shown to affect the organogenic potential of *Picea sitchensis* shoot tips (Selby and Harvey, 1985).

Including 5.37 nM NAA in the culture medium did not significantly affect survival of excised, cultured meristems (Table 5). In 1992, after 21 weeks in culture, 10.6% of meristems cultured on medium containing NAA were alive compared to 9.3% of meristems cultured on medium without NAA (over all forcing treatments). In 1993, after 21 weeks, 45.6% of the meristems cultured on medium without NAA were alive compared to 57.1% of the meristems cultured on medium containing NAA. Including 5.37 nM NAA in the culture medium also did not significantly affect the number of leaves produced by the meristems (Table 5). In 1992, surviving meristems on medium without NAA had a mean of 8.2 leaves compared to 11.7 leaves on meristems cultured for 21 weeks on medium with NAA (over all forcing treatments). In 1993, meristems cultured on medium without NAA had a mean of 11.1 leaves and meristems cultured on medium with NAA had 10.3 leaves.

Table 5. Effects of including 5.37 nM NAA in the culture medium on survival and the number of leaves produced per surviving eastern white pine shoot apical meristem after 21 weeks in culture in 1992 and 1993.

	Concentration of NAA in culture medium (nM)	
	0	5.37
Percent survival		
1992	9.3[1]	10.6
	(2.7)	(2.9)
1993	45.6	57.1
	(5.6)	(5.7)
Number of leaves		
1992	8.2	11.7
	(1.5)	(1.8)
1993	11.1	10.3
	(0.7)	(0.6)

[1]Each value is the mean (standard error of the mean) of three replications in 1992 and two replications in 1993 with 80 meristems per replication at the beginning of the experiment (forcing treatments combined).
[2]No values within a row were significantly different ($p \leq 0.05$) by LSD tests.

The absence of an effect of NAA in the two forcing experiments may have been the result of an insufficient concentration in the culture medium. However, we had previously tested higher concentrations (53.7 and 537 nM) and found that survival was not enhanced (although filters were not used) and meristems became disorganized and produced callus during development. Although we cannot rule out an intermediate auxin concentration that is beneficial, the apparent auxin independence of these meristems agrees with the reports for a number of other plants. *Dianthus caryophyllus* meristems required auxin and cytokinin only when no leaf primordia were present (Shabde and Murashige, 1977). The meristems in our study included 4-7 primordia and thus may not have required exogenous auxin. In addition, meristems of the conifer, *Sequoiadendron giganteum*, were successfully cultured without exogenous growth regulators when they were removed from the tree during budbreak (Monteuuis, 1991).

During the course of these and other experiments we have not observed any instances of meristems from

mature trees growing into shoots with fully juvenile characteristics. Meristems from seedlings grew rapidly, initiated primary leaves, underwent stem elongation, and in three cases formed adventitious roots without auxin treatment. The meristems from the 76-year-old trees exhibited a stable, mature developmental phenotype that was similar to development on intact branches. No truly juvenile primary leaves were produced. The primary leaves from the cultured mature meristems appeared to be cataphylls. The first cataphyll primordia developed into structures that resembled sterile scales. In some cases, subsequent primordia developed into modified leaves that subtended shoots, presumably brachyblasts. Both types of cataphylls became green and underwent some elongation, although only about 1/10 as much as a typical primary needle from a juvenile plant. Internode elongation was minimal in shoots derived from mature meristems, with the longest shoots attaining a length of 10 to 12 mm by the end of the 21-week culture period. In no cases, did shoots from mature meristems form roots.

Possible explanations for the lack of observed rejuvenation are that the clone studied is not prone to rejuvenation or that permissive culture conditions were not provided. One rejuvenated line was obtained from 300 excised meristems of *Sequoiadendron giganteum* trees (Monteuuis, 1991), but we have cultured several thousand meristems from branches of mature eastern white pines at various stages of growth over the past several years (results from 460 reported in this paper) without observing rejuvenation. However, further refinement of the culture conditions may facilitate expression of the juvenile developmental potential of mature white pine meristems. Another possibility is that the terminal meristem of a bud is highly fixed in its developmental pathway. Lateral meristems from brachyblasts may have greater potential for growing into shoots that exhibit juvenile characteristics than apical long shoot meristems (Abdullah et al., 1987; Browne, 1995). This tendency is exploited during hedging of stock plants to maintain rooting ability in pines (reviewed in Greenwood and Hutchison, 1993). Finally, rejuvenation, if possible in this species, may require a treatment in addition to removing the mature influence of surrounding tissue. A juvenility promoting effect might be obtained by grafting meristems onto juvenile rootstock as has been reported for meristems of *Pinus pinaster* (Monteuuis and Dumas, 1992) and shoot tips of *Sequoia sempervirens* (Huang et al., 1992).

Our results suggest that, in eastern white pine, removal of the meristem from surrounding tissues may not be sufficient for meristem rejuvenation. This differs from shoot meristems of maize that revert fully to the juvenile state when excised (Irish and Nelson, 1988). Thus, some conifers may have mechanisms for maintaining maturation states that are not utilized by herbaceous plants that undergo vegetative maturation. This stable, mature developmental state continues to be a principal obstacle to vegetative propagation of genetically superior conifers (reviewed in Bonga and von Anderkas, 1993).

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Short communication

In vitro shoot apex micrografting of mature *Acacia mangium*

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Key words: grafting, shoot tip, tissue culture, vegetative propagation

Abstract. Prospects for *in vitro* micrografting shoot apices of mature *Acacia mangium* trees were investigated with the use of 432 micrografts. Overall success rates of 51.5% were obtained for shoot apices ranging from 200 to 400 µm in length, and a short basal wedge of underlying tissues top-grafted in aseptic conditions onto 2-to-3-month-old *in vitro* grown *Acacia mangium* seedlings. The successfully established micrografts displayed, however, substantial variability in terms of further scion elongation as 41% of these micrografts, or 21.2% only of the total amount of the micrografts performed, had resumed growth two months after micrografting. The elongated scions exhibited different types of morphology, ranging from juvenile-like type composed leaves to the predominant mature-like phyllode morphology. Side-grafting, a more difficult procedure to perform than top-grafting, or placing the micrografts for 2 weeks in darkness after grafting, did not improve the scores. Moreover, attempts to micrograft meristems (150–200 µm) resulted in 5% success only.

Introduction

Acacia mangium Willd. is a pioneer legume tree which has gained an increasing interest for use in (re)afforestation in many tropical countries, especially for pulpwood production in South-East Asia. This is mainly due to its remarkable growth potential, particularly on very acid and poor soils. The fertility under these conditions can be improved due to its nitrogen fixing ability. Tissue culture is likely to play an important role in tree improvement, and possibly in development programmes of *Acacia mangium*, provided that a suitable technology is available. Although started recently, micropropagation of mature selected trees has been so far hindered by firstly in obtaining axenic cultures free of endogenous bacteria, and secondly, in obtaining rejuvenated explants that display a satisfactory potential for micropropagation compared to juvenile plant material (Pierik, 1990). It appears from the literature (Jonard, 1986; Hartmann et al., 1990; Pierik, 1990) that if the shoot tips used as scions are small enough, *in vitro* micrografting may overcome these problems by regenerating pathogen-free and possibly rejuvenated tissues (Jonard, 1986). The prospects of micrografting *in vitro* shoot apices from mature *Acacia mangium* trees were therefore investigated and are reported in this paper.

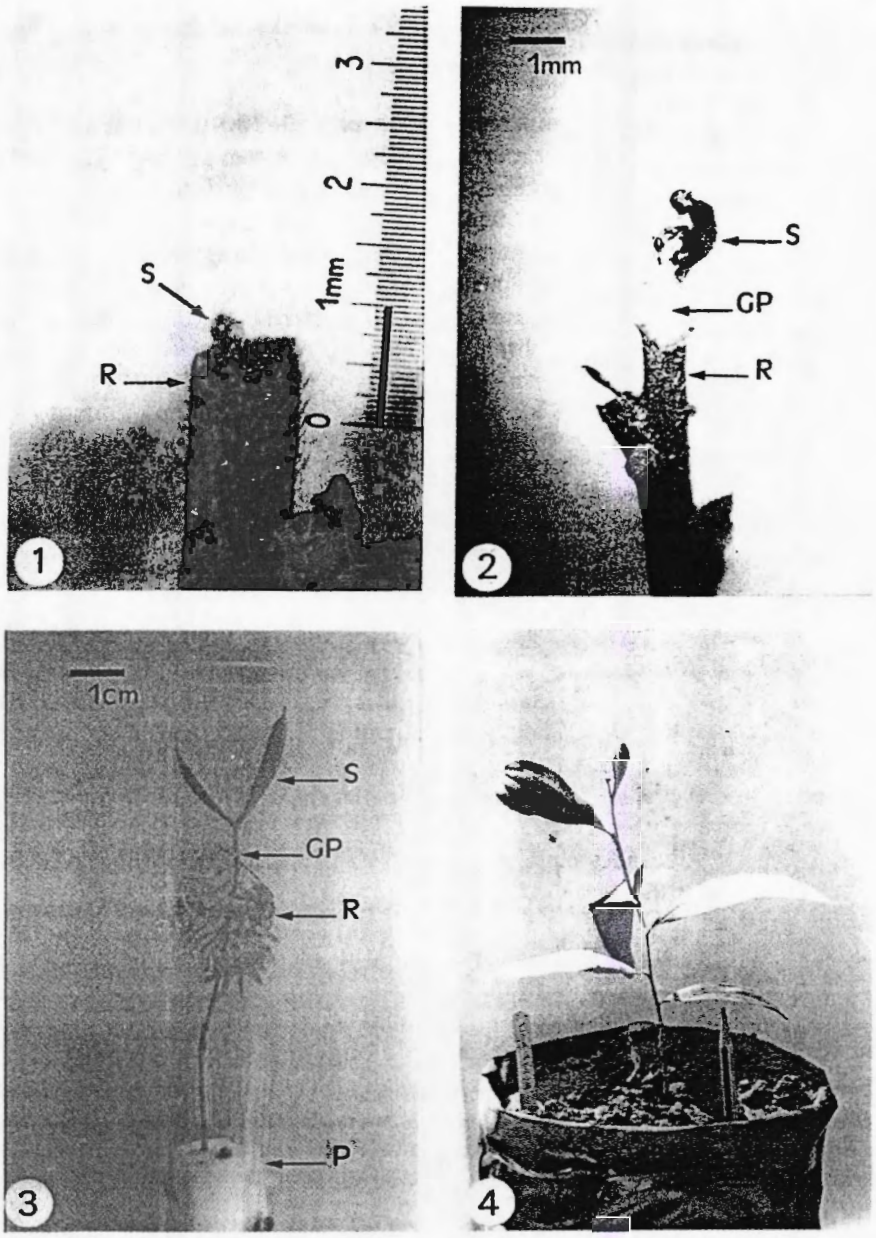
Materials and methods

The *Acacia mangium* seeds of Papua New-Guinea origin and used for the production of *in vitro* rootstocks were first soaked for 5 to 10 sec in boiling water, then immersed for 5 min in 70% ethanol and finally in 1‰ $HgCl_2$ aqueous solution. After three thorough rinses in sterilized ultrapure water, the seeds were placed individually on 20 × 30 mm 'Sorbarod' cellulosic plugs in 21 × 150 mm glass test tubes covered with polypropylenne caps. The plugs had been previously saturated with 5 ml liquid medium consisting of half-strength macro and micronutrients described by Murashige and Skoog (1962), 20 g l^{-1} sucrose and with pH adjusted to 5.5–5.6 prior to autoclaving at 120 °C and 95 kPa for 20 min. The advantage of using such plugs is the possibility to draw the rootstock out of the tube without damaging its root system. This makes the micrografting manipulations easier. Cultures were maintained under a 16-h photoperiod (50–60 µmol m^{-2} s^{-1}, 'TLD 36W/84 Philips' fluorescent lamps) at 28/22 ± 2 °C light/dark. Under these conditions, 60 to 80% of the seeds germinated to develop, within 2 to 3 months, into 3 to 4 cm tall young seedlings with elongating epicotyl suitable for grafting.

The shoot apices used as scions originated from shoot tips were collected from the lower part of the crown of 5- to 12-year-old *Acacia mangium* trees growing outdoors in Sabah (East Malaysia). All the grafting manipulations described hereafter were performed under a laminar flow hood and in aseptic conditions. Immediately after the collection, the shoot tips were dipped in 70% ethanol for 5 min, rinsed in sterilized ultrapure water and then wrapped in pure water moistened tissue paper before dissection under a binocular microscope with cold-light source. Unless otherwise stated, the shoot apices were top-grafted according to the following technique. After the removal of the outer young phyllodes, the shoot apex, ranging from 200 to 400 µm (0.2 to 0.4 mm) in length, plus a short 'V' shaped basal wedge of the underlying tissues, was excised and quickly but carefully inserted into a small vertical split made in the centre of the top cut surface. Such a surface resulted from the decapitation of the rootstock epicotyl which resembled a miniaturized 'cleft-graft' (Hartmann et al., 1990) with scions that did not exceed 800 µm in length (Figure 1). Once grafted, the seedlings were returned to the previously described environmental conditions. Callus formation needed to maintain the scion alive before it could undergo new organogenesis started a few days after grafting. From this stage onwards, any of the numerous axillary shoots produced by the stock was systematically removed to avoid competition.

The influence of three different factors on micrografting success was investigated in a greater depth. These factors were:

1. the micrografting technique, comparing the basic top-grafting routinely used in the course of this work to the side-grafting technique consisting of inserting the scion into a lateral cut made on the side of the stock epicotyl (Monteuuis, 1986) instead of its decapitated top;



Figures 1–4. Successive developmental stages of shoot apices from mature *Acacia mangium* trees micrografted *in vitro*. (1) Shoot apex (S) newly top-grafted in aseptic conditions onto an *in vitro* grown seedling rootstock (R). (2) Elongation scion (S) with its 'V' shaped basal wedge connected to the rootstock (R) tissues at the grafting point (GP). (3) *In vitro* micrograft showing above the grafting point (GP) the developing scion (S) with mature-like phyllode morphology which contrasts with the juvenile composed leaves of the seedling rootstock (R) cultivated on the 'Sorbarod' plug (P). (4) Micrograft derived from a 12-year-old donor tree after acclimatization to outdoor conditions (pencil as scale).

2. the effect of placing the micrografts for 2 weeks in darkness just after micrografting and before transferring them to the standard lighting conditions;
3. the size of the scion, comparing scions of 150–200 µm in length, that corresponds to the apical meristem plus some youngest phyllodes in addition to a small basal wedge, to the standard apex scions.

These experiments were conducted separately, involving samples each consisting of 12 micrografts and repeated for each treatment at different dates. Experimental treatments were tested for significance ($P \leq 0.05$) using the Chi-square test (Snedecor and Cochran, 1957).

Results and discussion

A total of 432 shoot apices from mature *Acacia mangium* trees were micrografted during the course of the study. Forty-three micrografts, i.e. 10% (43/432) of the total, were lost due to fungal contamination and were therefore excluded from the analyses shown.

Out of a total of 293, 151 to-grafted shoot apices micrografted remained alive 2 months after grafting, demonstrating that a graft union between the stock and the scion had occurred and the micrografts could be considered as successfully established. This number corresponded to an overall success rate of 51.5%, despite a great variability in terms of the scion further expansion, as already observed for other species (Monteuuis, 1987, 1994). As an indication, 2 months after grafting only 41.1% (62/151) started to elongate (Figure 2), the remainder being capable to rest for months without any sign of potential for further growth.

Side-grafting the scion is a more difficult procedure to perform and it induces a starting angle of the scion in contrast to the top-grafting technique routinely practised. In this study, however, it did not improve significantly the overall proportion of the successfully established micrografts (57/96 = 59.4% vs. 50/92 = 54.3%, respectively), or the elongating scions (19/57 = 33.3% vs. 21/50 = 42.0%, respectively after 2 months) contrary to that observed for Douglas-fir (Monteuuis, 1995).

Regardless of the technique used, placing the micrografts for 2 weeks in darkness just after grafting resulted in fewer successful grafts than the control kept in standard lighting conditions (5/19 = 26.3% vs. 11/20 = 55%, respectively). This procedure, which also inhibited scion elongation, is in contrast to that reported for *Picea abies* (Monteuuis, 1994).

Attempts to restrict the scion to the apical meristem plus some of the youngest phyllodes (150 to 200 µm as overall scion size) led to a tremendous decrease in the success rate (2/36 = 5.6% vs. 21/33 = 63.6% for the standard size apices, $P < 0.001$) due to an early higher mortality, as observed for other species (Jonard, 1986; Monteuuis, 1987), and also to excessive callus

proliferation from the stock that choked the grafted meristem before it could resume growth. Noticeably, the only grafted meristem that further developed gave rise to a shoot with composed leaves or pinnates that characterized the juvenile stage, in contrast to the phyllodes of the adult phase of this heteroblastic species (Doorenbos, 1965), whereas shoots emerging from micrografted apices exhibited overall a larger proportion of phyllodes (Figure 3). Further experiments are needed at this stage to establish the influence of the scion size on the possibility to induce rejuvenation through micrografting in *Acacia mangium*.

In addition to these rejuvenation prospects, the micrografting technique described was found to be helpful for reducing contamination problems which pose severe limitations in an efficient micropropagation process, particularly when mature genotypes of *Acacia mangium* are desired.

Although success may vary according to the dexterity of the manipulator, and notwithstanding the substantial variability in terms of the scion further elongation under the experimental conditions described above, the technique developed can henceforth be considered as a helpful alternative to *in vitro* apex or even meristem culture of mature *Acacia mangium* which is still problematic, while combining the advantages of grafting (Hartmann et al., 1990). The *in vitro* micrografts produced can be further handled as high quality stock plants either in *in vitro* or *in vivo* conditions after a successful acclimatization (Figure 4) that is facilitated by the 'Sorbarod' plug used as stock support.

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Ex Vitro Survival, Rooting and Initial Development of *in Vitro* Rooted *vs* Unrooted Microshoots From Juvenile and Mature *Tectona grandis* Genotypes

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Summary

The influence of *in vitro* formed adventitious roots on acclimatization and initial *ex vitro* development of microshoots from juvenile and mature teak (*Tectona grandis*) genotypes was investigated. Overall, the *in vitro* rooted microshoots gave rise to higher survival and *ex vitro* rooting rates 7 weeks after transfer than those not rooted *in vitro*. The age difference resulted in higher mortality rates 7 and 15 weeks after transfer for the microshoots of mature origin. The number of roots produced *in vitro* was observed to have a strong influence on the number of roots formed *ex vitro* 7 weeks after transfer and on the height of the microshoots at the time of transfer, 7 weeks later and to a lesser extent after 15 weeks. Differences in height at transfer between microshoots from the two origins of plant material tended to disappear during the acclimatization process. Overall, more than 80% of the microshoots that were initially transplanted from the various categories tested were successfully acclimatized to *ex vitro* conditions. These results are discussed considering mainly the influence of the maturation process on the formation of *ex vitro* roots in *in vitro*-derived microshoots and emphasizing the role of some basic exogenous factors.

Key words: acclimatization, adventitious rooting, age, *ex-vitro* development, microshoots, *Tectona grandis*.

FDC: 165.44; 181.65; 176.1 *Tectona grandis*.

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Résumé

L'influence de l'appareil racinaire de type adventif formé *in vitro* sur l'acclimatation et les premiers stades de développement *ex vitro* de microboutures de génotypes juvéniles et mature de teck (*Tectona grandis*) a été etudiée. Globalement, 7 semaines après leur transfert, les microboutures enracinées *in vitro* se distinguent par des taux de survie et d'enracinement *ex vitro* supérieurs à leurs homologues non enracinées *in vitro*. L'influence de l'âge se ressent au niveau des taux de mortalité plus élevés 7 et 15 semaines après le transfert pour les microboutures provenant du clone mature. Le nombre de racines formées *in vitro* influe sur le nombre de racines développées en conditions *ex vitro* 7 semaines après le transfert, ainsi que sur la hauteur des microboutures à la date du transfert, après 7 semaines, et dans une moindre mesure après 15 semaines. Les différences de hauteur mises en évidence entre les deux origines à la date du transfert tendent à s'estomper durant l'acclimatation. Plus de 80% de l'ensemble des microboutures transférées *ex vitro* ont pu être acclimatés avec succès. Ces résultats sont discutés en considérant principalement l'impact du phénomène de maturation sur la rhizogenèse *ex vitro* de microboutures issues d'*in vitro*, ainsi que l'influence de certains facteurs exogènes prépondérants.

Mots clés: acclimatation, âge, développement *ex-vitro*, enracinement adventif, microboutures, *Tectona grandis*.

Introduction

Tectona grandis, commonly known as teak, has gained a worldwide reputation as a high quality timber on account of the attractiveness and durability of its wood. This arborescent species occurs naturally although discontinuously in deciduous

forests between 9° to 26° North latitude, and 73° to 104° East longitude, including central and southern India, Myanmar, northern Thailand and Laos. It has been subsequently introduced to many south-east Asia countries such as Indonesia, Sri-Lanka, Vietnam, West and East Malaysia, Solomon Islands and to some African countries like Ivory Coast, Nigeria and Togo. In 1990, the species was reported to cover about 25 millions ha, but the resources currently available are still far below the huge market demand. Planting stock is still produced from seeds despite certain handicaps such as quantitatively limited and late seed production, low germination rates, substantial variability in growth and wood quality among individuals within progenies (WHITE, 1991).

These limitations prompted us to explore whether mature selected genotypes could be clonally mass propagated true-to-type using nursery and tissue culture protocols (MONTEUUIS, 1995; MONTEUUIS et al., 1995b). For *in vitro* production, success is highly dependent upon the acclimatization process. In this respect, the formation of adventitious roots from a microshoot is the limiting factor (MCCLELLAND et al., 1990). The rooting process can occur *in vitro* or *ex vitro* depending on the species and the facilities available (DRIVER and SUTTLE, 1987).

The aim of this work was to compare *in vitro* rooted and unrooted microshoots of juvenile and mature teak genotypes with regard to their capacity to be successfully acclimatized to *ex vitro* conditions, to produce *ex vitro* roots and to resume early growth, considering the relative lack of accurate data available from the literature on this subject (MASCARENHAS et al., 1987; MASCARENHAS and MURALIDHARAN, 1993).

Material and Methods

Plant material origin and in vitro culture conditions

Microshoots from 2 different age origins of *Tectona grandis* were compared. The mature plant material was obtained from 1 cm to 2 cm long nodal explants collected from elongating shoots from the lower part of the crown of a 15 year-old *Tectona grandis* ortet growing outdoors at Luasong Forestry Centre, Sabah, East Malaysia. Juvenile explants were obtained from *in vitro* germinated seeds collected from the same ortet. Both origins systematically paired during all culture procedures were propagated through 1 cm to 2 cm long nodal microcuttings during 1 year alternating every 1.5 month subcultures on a basal multiplication and elongation culture medium (MONTEUUIS, 1995; BON and MONTEUUIS, 1996). This medium was solidified with 7gl^{-1} "high gel strength" Sigma agar after pH adjustment to 5.5 to 5.6, then autoclaved at 120 °C and 95 kPa for 20 min. All the cultures were maintained in 21 mm x 150 mm glass test tubes containing 10 ml of culture medium and covered with polypropylene caps under a 16-h photoperiod (50 to 60 µmol m^{-2}s^{-1}, "TLD 36W/84 Philips" fluorescent lamps) at 28/22 °C ± 2 °C light/dark. Transfer of the microshoots to *ex vitro* conditions occurred after 1.5 month of culture on the basal elongation medium where a large proportion of microshoots had already rooted spontaneously *in vitro*.

Ex vitro conditions

The 2 cm to 3 cm tall *in vitro* microshoots selected for the experiments (see hereunder for more information) were removed from the culture tubes and soaked for a few minutes in an aqueous fungicide solution – Thiram 80, 5g/L – before being inserted according to the experimental design into the rooting beds. Beds were filled with wet sand used as rooting substrate after it had been boiled to reduce disease risks.

Once set into the rooting bed, the microshoots were maintained under 50% shade with intermittent-mist water sprays provided by a mist system, the frequency of which was controlled by an "electronic leaf" system (HARTMANN et al., 1990) to avoid any desiccation damage. Aqueous fungicide solutions – mainly Thiram 80, 5 gl^{-1} – were sprayed on the microshoots weekly.

After 7 weeks the plantlets were carefully removed from the rooting substrate and potted individually in 10 cm x 15 cm black plastic bag containers filled with clayish local top soil, then maintained in the same *ex-vitro* conditions as described previously, except that the frequency of the misting was halved. The potted plants were kept 8 weeks under this regime, then removed from the mist-system to the field nursery.

Experimental designs, analysis criteria and statistical treatment of the data

Experiment 1

Survival capacity and rootability of *in vitro* rooted versus not rooted microshoots from juvenile and mature origins after transfer to *ex vitro* conditions were analyzed adopting a full factorial design involving the four combinations. Three contiguous randomized complete blocks were established, each including 4 elementary plots of 20 microshoots by combination. This experiment was first done on 9th of May 1995, then repeated using similar experimental conditions on the 10th of October 1995. This resulted in a total of 20 x 2 x 2 x 3 x 2 = 480 microshoots. Seven weeks after transfer into the rooting beds, the surviving microshoots were recorded according to their origin, distinguishing between the rooted and not rooted plantlets to establish the corresponding *ex vitro* rooting rate out of the microshoots still alive and the survival rates, based on the number of microshoots initially transferred to the rooting bed. Survival was recorded again for each plant material combination 8 weeks after potting (15 weeks after the date of transfer to *ex vitro* conditions), just before the potted plantlets were moved to the nursery.

The data were analyzed using the SPSS statistical package (SPSS inc., 1990). Null hypotheses were rejected for probability value $P \leq 0.05$. Tests for homogeneity of variance were performed using CROCHRAN's and BARTLETT's tests (SPSS Inc., 1990) which established the suitability of transforming the survival and rooting rates by arcsin. One-way analysis of variance, followed by the Least Significant Difference test "LSD" when the null hypothesis was rejected, was used to assess the influence of the different categories of plant material on survival and *ex-vitro* rooting and then to compare the corresponding means (SPSS Inc., 1990). The effects of the experimental factors on the same traits were assessed carrying out an analysis of variance, ANOVA (SPSS Inc., 1990), of the following model:

$$Y_{ijkl} = \mu + R_i + O_j + D_k + B_l + (RO)_{ij} + (RD)_{ik} + (RB)_{il} + (OD)_{jk} + (OB)_{jl} + (DB)_{kl} + \varepsilon_{ijkl}$$

where:

Y_{ijkl}: value of the plot submitted to the i^{th} level of factor *in vitro* "rooting", j^{th} level of factor "origin", k^{th} level of factor "date of experiment" and l^{th} level of factor "block";

μ: "grand mean" of the whole experiment;

R_i: effect of the factor *in vitro* "rooting", $1 \leq i \leq 2$;

O_j: effect of the factor "origin" of the microshoots, $1 \leq j \leq 2$;

D_k: effect of the factor "date", $1 \leq k \leq 2$;

B_l: effect of the factor "block", $1 \leq l \leq 3$;

$(RO)_{ij}$: effect of the interaction between the factors "rooting" and "origin";

$(RD)_{ik}$: effect of the interaction between the factors "rooting" and "date";

$(RB)_{il}$: effect of the interaction between the factors "rooting" and "block";

$(OD)_{jk}$: effect of the interaction between the factors "origin" and "date";

$(OB)_{jl}$: effect of the interaction between the factors "origin" and "blocks";

$(DB)_{kl}$: effect of the interaction between the factors "date" and "blocks";

ε_{ijkl}: random error.

Experiment 2

The influence of the number of roots formed *in vitro* on survival, root production and growth of the microshoots after transfer to *ex vitro* conditions was examined by selecting for each origin 10 *in vitro* microshoots for each of the following classes: no root, 1 root, 2 roots, 3 roots (restricted to 5 microshoots from the mature origin), and 4 roots or more (from the juvenile origin only as none was available from the mature origin). These microshoots were transferred to the rooting bed in a completely randomized single-microcutting plot design, with a regrouping of the juvenile and the mature origins together and individually identified with a label. In addition to the survival rates defined previously, records were made for each labelled plantlet in order to determine the following criteria: (*i*) the height (H0) of the plantlet just after setting in the rooting bed; (*ii*) the height at 7 weeks (H7) and (*iii*) the number of roots (NRex) developed after 7 weeks in the rooting bed and just prior to potting; (*iv*) the height H8 just after potting; (*v*) the height H15 8 weeks after potting (15 weeks from the date of transfer to *ex vitro*) and before dispatch to the nursery; (*vi*) the height increment HI1 7 weeks after transfer to *ex-vitro* conditions (HI1 = H7 − H0); (*vii*) the height increment HI2 8 weeks after potting (HI2 = H15 − H8) and (*viii*) the difference DNR between the number of roots *in vitro* just before transfer and the number of roots recorded *ex vitro* 7 weeks later.

This second experiment was conducted under the same experimental conditions and on the same dates as Experiment 1. A total of 170 microshoots, $10 \times 5 \times 2 = 100$ for the juvenile genotypes and $(10 \times 3 \times 2) + (5 \times 2) = 70$ for the mature clone were observed. The influence of the different categories of plant material on the various traits observed and the relevant mean comparisons were analyzed applying the same statistical procedures as described for Experiment 1, with data transformed by $\log_{10}$ to fulfil homogeneity of variance requirements when needed. Except for the survival rates for which the Chi-square test was used (SPSS Inc., 1990), the effect of the different experimental factors on the various traits observed was assessed through an analysis of variance of the following model:

$$Y_{ijk} = \mu + N_i + O_j + D_k + (NO)_{ij} + (ND)_{ik} + (OD)_{jk} + \varepsilon_{ijk}.$$

where:

Y_{ijk}: value of the plot corresponding to the i^{th} level of factor "number of *in vitro* roots", the j^{th} level of factor "origin" and the k^{th} level of factor "date of experiment";

μ: grand mean;

N_i: effect of the factor "number of *in vitro* roots" of the microshoots, $1 \leq i \leq 4$;

O_j: effect of the factor "origin", $1 \leq j \leq 2$;

D_k: effect of the factor "date", $1 \leq k \leq 2$;

$(NO)_{ij}$: effect of the interaction between the factors "number of *in vitro* roots" and "origin";

$(ND)_{ik}$: effect of the interaction between the factors "number of *in vitro* roots" and "date";

$(OD)_{jk}$: effect of the interaction between the factors "origin" and "date";

ε_{ijk}: random error.

Due to imbalance in the data between the 2 origins of plant material, the microshoots with 4 roots or more developed *in vitro* were excluded from the analysis of variance.

Results

Experiment 1

The 4 categories of microshoots were found to noticeably influence the *ex vitro* rooting rate ($P = 0.001$) and survival scores at 7 weeks ($P = 0.009$) and 15 weeks ($P = 0.010$) after transfer from *in vitro* as illustrated in *figure 1*. The analysis of variance (*Table 1*) established that the *in vitro* rooting status of the microshoots prior to acclimatization had an influence on survival after 7 weeks, to the advantage of the microshoots rooted *in vitro* compared to the not rooted ones (93.8% vs 85.46%, respectively). However, this effect was no longer significant 15 weeks after transfer. *In vitro* rooted microshoots also gave rise to higher *ex vitro* rooting rates than those transferred from *in vitro* without roots (98.4% vs 83.7% respectively). Differences in age origin of the microshoots resulted in significant differences in survival 7 weeks and 15 weeks after transfer to *ex vitro* conditions (*Table 1*), with higher scores for the juvenile plant material compared to the mature clone (95.4% vs 83.7%, and 94.2% vs 81.3%, respectively). A significant interaction between the rooting status *in vitro* and the age origin was found only for the *ex vitro* rooting rates (*Table 1*), with the greatest difference observed in that respect between the *in vitro* rooted versus unrooted microshoots of the mature origin, as shown in *figure 1*.

Table 1. – Results from the analyses of variance (significance levels, F-test) performed for the various traits investigated in relation to the different experimental factors assessed in Experiment 1 (see text for more information).

Source of variation	df	Traits		
		Surv.7	Surv.15	Root.
In vitro rooting status (R)	1	0.036*	0.071	0.001***
Origin (O)	1	0.005**	0.010**	0.664
Dates (D)	1	0.253	0.810	0.741
Blocks (B)	2	0.910	0.933	0.069
R X O	1	0.641	0.419	0.024*
R x D	1	0.063	0.100	0.563
R x B	2	0.251	0.347	0.762
O x D	1	0.229	0.364	0.125
O x B	2	0.213	0.226	0.059
D x B	2	0.347	0.434	0.757

Surv. 7 and Surv. 15: survival rates of the microshoots respectively 7 weeks and 15 weeks after transfer; Root.: rooting rates 7 weeks after transfer. The data were transformed by arcsin for homogeneity of variance requirements. Asterisks indicate a significant effect of the experimental factors on the trait considered at *) $P \leq 0.05$, **) $P \leq 0.01$ and ***) $P \leq 0.001$ levels of significance.

Experiment 2

The different categories of microshoots assessed influenced the height of the plant material (*i*) at the time of transfer to *ex vitro* conditions (HO, $P < 0.001$), (*ii*) after a period of 7 weeks (H7, $P < 0.001$), and (*ii*) after 15 weeks (H15, $P = 0.004$). Microshoots without any roots *in vitro*, or those with only one root from the juvenile origin were significantly shorter than the other categories at the time of transfer, as illustrated in *figure 2*. This trend was still present 7 weeks after transfer, whereas the average height of the juvenile microshoots not rooted *in vitro* was comparable to those of the other categories after 15 weeks (*Figure 2*). Height increment following transfer was not significantly influenced by the different microshoot categories, unlike the number of *ex vitro* roots ($P = 0.002$). For this latter criterion, *figure 3* shows that the highest scores were obtained for the juvenile microshoots which had formed 3 and 4 roots *in vitro*.

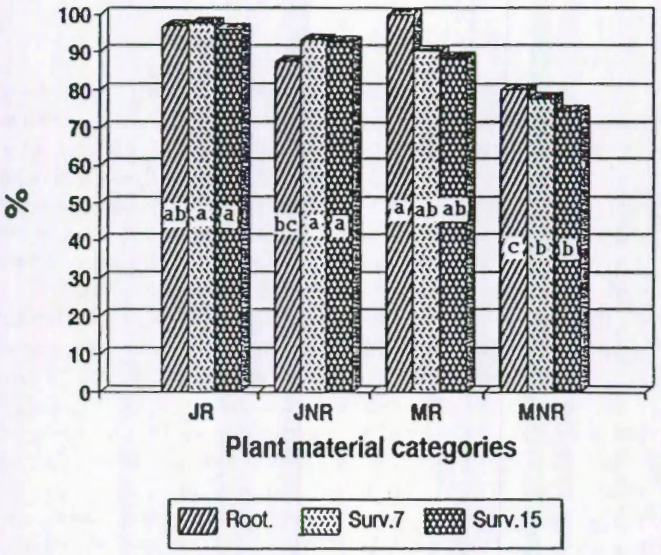

Figure 1. – Mean ex-vitro rooting rates (Root.) and survival rates 7 weeks (Surv. 7) and 15 weeks (Surv. 15) after transfer to ex-vitro conditions of the 4 categories of microshoots assessed in Experiment 1, i.e. juvenile rooted in vitro (JR), juvenile not rooted in vitro (JNR), mature rooted in vitro (MR) and mature not rooted in vitro (MNR). For each of the 3 criteria observed, letters distinguish means which are significantly different at the 5% level (LSD test).

Results from the analysis of variance considering each experimental factor investigated independently of the others are given in *table 2*. The height of the microshoots was strongly influenced by the number of roots formed *in vitro*, at the time of transfer, 7 weeks later and to a lesser although still significant extent after 15 weeks, which was consistent with the significance levels observed for HI1 and HI2. Overall, the microshoots from the mature origin were taller than the juvenile ones at the time of transfer (1.76 cm vs 1.42 cm). This difference in height between the 2 origins was still present but less obvious after 7 weeks (3.38 cm vs 3.20 cm and disappeared after 15 weeks. The date of transfer noticeably affected the height of the microshoots *ex vitro* recorded after 7 weeks, as well as the height increment measured at that time. The number of *ex vitro* roots was markedly influenced by the number of roots formed previously *in vitro*, with a significant date effect pointed out also for the difference (DNR) between the number of roots formed *in vitro* and those produced *ex vitro* (*Table 2*). Overall, higher scores were obtained for the microshoots transferred in May (data not shown).

Interactions between the age origin of the microshoots and the number of roots produced *in vitro*, which was most obvious for the microshoots with one root *in vitro* as shown in *figure 2*. were found significant only for height after 7 weeks and 15

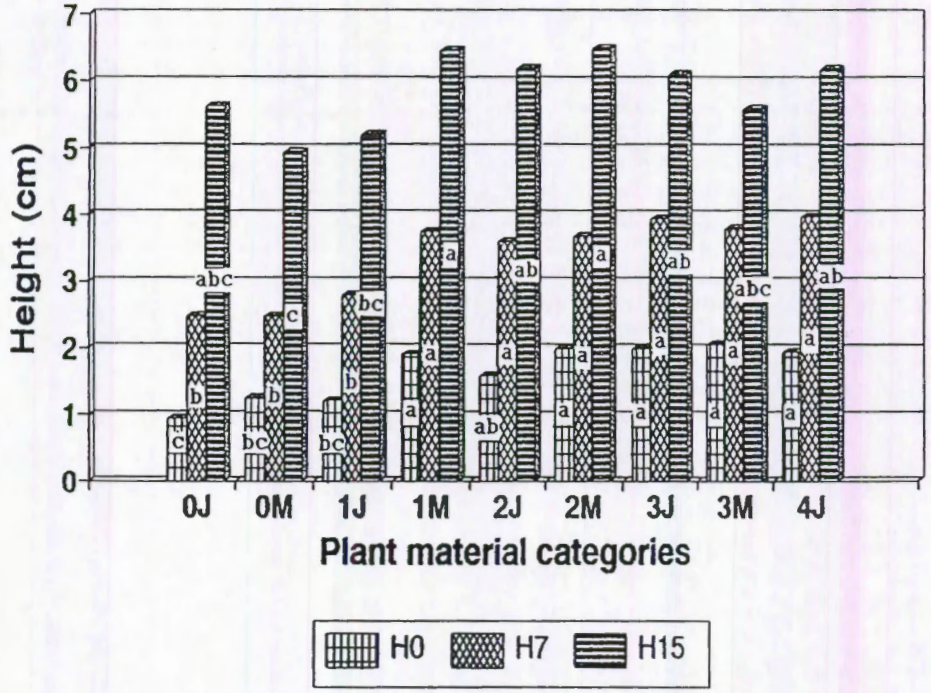

Figure 2. – Mean height measurements *i*) just after transfer HO, *ii*) 7 weeks after transfer H7 and *iii*) 15 weeks after transfer H15 (8 weeks after potting) to ex-vitro conditions of the different categories of microshoots assessed in Experiment 2, i.e. without any root in vitro from juvenile (0J) and mature (0M) origins, with 1 root in vitro from juvenile (1J) and mature (1M) origins, with 2 roots in vitro from juvenile (2J) and mature (2M) origins, with 3 roots in vitro from juvenile (3J) and mature (3 M) origins and with 4 roots in vitro from juvenile (4J) origin. For each of the 3 heights considered, letters distinguish means which are significantly different at the 5% level (LSD test).

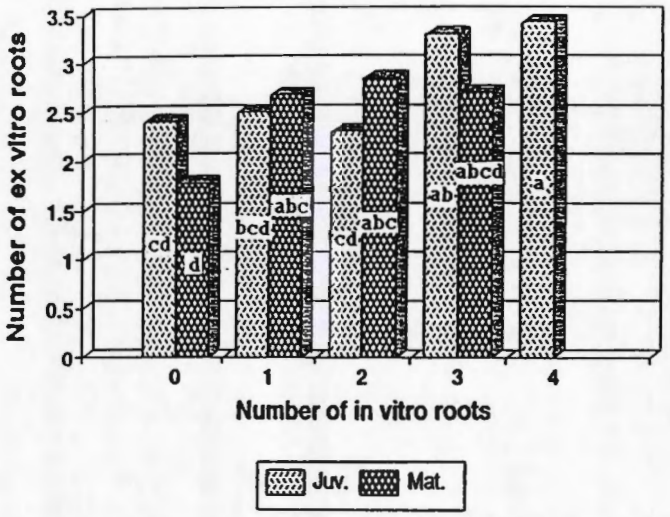

Figure 3. – Average number of ex-vitro roots formed by the microshoots 7 weeks after transfer, in relation to their age origin and the number of in vitro roots they had at the time of transfer in Experiment 2. Letters distinguish means which are significantly different at the 5% level (LSD test).

weeks. Corresponding height records, as well as height increment measurements after 7 weeks (HI1), were observed to be much higher overall for the microshoots of the juvenile origin transferred in May compared to the other origin X date combinations, as reflected by the significant relevant interactions (*Table 2*).

Discussion

The practical prospects of mass micropropagation are largely dependent on the success of the acclimatization process and the capability of the acclimatized plantlets to resume growth. In that respect and based on previous observations, it seems founded to devote special attention to the *in vitro* rooting status of the microshoots prior to acclimatization, which is liable to vary according to the age of the plant material (HACKETT, 1985, 1988) and to the genotype (HAISSIG and RIEMENSCHNEIDER, 1988). Genetically closely related mature and juvenile plant material origins were utilized in order to minimize the genetic effects.

Adventitious roots formed *in vitro* have been reported for different species to display particular morphological and anatomical features induced by the physical characteristics of the gelled culture medium (MOHAMMED and VIDAVER, 1988; MCCLELLAND and SMITH, 1988, MCCLELLAND et al., 1990). Such roots may stimulate growth *in vitro* as established by the measurements made at the time of transfer but in the same way as noticed for other tree species (MCCLELLAND et al., 1990) *in vitro* formed roots did not seem to be adapted to more natural conditions. Indeed, most of them were observed to disappear after transfer to the sand used as rooting substrate, to be replaced by more slender, branched *ex vitro* produced roots with whitish tips. These roots were assumed to be more functional than those formed *in vitro* (MCCLELLAND et al., 1990). When originating from root tissues initiated *in vitro*, the new *ex vitro* roots must be considered from the anatomical standpoint, more as a result of a root regeneration process than as a *de novo* formation of adventitious roots from shoot tissues depending upon the capability of the latter to dedifferentiate (HARTMANN et al., 1990). This basic distinction of the origin of the roots developed *ex vitro* could account for the differences observed after transplanting between the *in vitro* rooted and unrooted microshoots as regards the *ex-vitro* rooting rates especially when referring to the mature origin. Adventitious rooting has been reported to be more difficult to achieve from mature tissues than from juvenile ones (HACKETT, 1988). *In vitro* conditions have also been proven for different woody species to be more suitable for rooting shoots of mature origin than *ex vitro* conditions (FRANCLET, 1983; HACKETT, 1988; MCCOWN, 1988). This could be due to the possibility to rejuvenate the mature tissues through successive transfers onto appropriate *in vitro* media, thereby inducing a progressive enhancement of their potential for adventitious rooting (FRANCLET, 1983; HACKETT, 1985, 1988). At the time of transplant however, some of the microshoots of the mature origin had not rooted spontaneously *in vitro*, possibly because they were not sufficiently rejuvenated to display the same capability for *ex vitro* adventitious rooting as their homologs from the juvenile origin. This lesser ability for adventitious root formation after transfer to *ex vitro* conditions likely induced higher mortality rates as compared to the juvenile origin. Thus, for this kind of plant material, it seems advisable to root the microshoots *in vitro*, prior to their transfer to *ex vitro* conditions. Another option which may be worth testing is to dip the base of the microshoots not rooted *in vitro* into a rooting powder prior to setting in the rooting beds, as recommended for *Sequoia sempervirens* (POISSONNIER et al., 1980). Depending on the species, treatment with exogenous auxin can stimulate the potential for *ex vitro* rooting, while avoiding an additional transfer of the microshoots onto a special *in vitro* rooting medium. Practically, *ex vitro* rooting of tissue-cultured microshoots offers basic advantages and results in a substantial gain of money (MCCOWN, 1988).

Table 2. – Results from the analyse of variance (significance levels, F-test) performed for the various traits investigated (see text for definition) in relation to the different experimental factors assessed in Experiment 2.

Source of variation	df	Traits						
		H0	H7	H15	HI1	HI2	NRex	DNR
No of roots in vitro	3	<0.001***	<0.001***	0.026*	0.003**	0.111	0.001***	0.070
Origin	1	<0.001***	0.030*	0.537	0.158	0.177	0.192	0.373
Dates	1	0.442	<0.001***	0.391	<0.001***	0.255	<0.001***	0.002**
No of roots in vitro X Origin	3	0.126	0.048*	0.029*	0.138	0.363	0.236	0.248
No of roots in vitro X Dates	3	0.079	0.332	0.282	0.591	0.322	0.008	0.092
Origin X Dates	1	0.635	0.007**	0.028*	0.003*	0.474	0.453	0.380

H0, H7, NRex and DNR data were transformed by $\log_{10}$ for homogeneity of variance requirements.
Asterisks indicate a significant effect of the experimental factors on the trait considered at *) $P \leq 0.05$, **) $P \leq 0.01$ and ***) $P \leq 0.001$ levels of significance.

As far as the influence of exogenous factors on *ex vitro* adventitious rooting is concerned (RAUTER, 1983), special consideration has to be devoted to the mist system (MCCOWN, 1988; MOHAMMED and VIDAVER, 1988; DEBERGH, 1991). Use of mist enabled us to maintain the survival of the different categories of microshoots, especially those not rooted *in vitro*, for the time needed to develop *ex vitro* roots and to get them finally acclimatized with overall success rates of more than 80%. This score is far superior to the data available so far from the literature in the same field on the same species based on the use of a specific *in vitro* rooting medium (MASCARENHAS et al., 1987). The protocol developed in our conditions has been useful for successfully acclimatizing 50.000 microshoots of teak to date, mainly from mature genotypes. Another important factor with regard to adventitious rooting is the rooting substrate (RAUTER, 1983; HARTMANN et al., 1990). Sand was used as it was locally more easily available than other components which might have been more appropriate to increase the rooting rates and consequently the acclimatization success (MOHAMMED and VIDAVER, 1988; HARTMANN et al., 1990). Light has been also observed to influence adventitious root formation differently according to the species (THOMPSON, 1992). In our case, longer photoperiod in May could have stimulated the formation of *ex vitro* roots and consequentially the early growth of the microshoots compared to those transferred in October. Conversely, under the same experimental conditions, the potential for adventitious root formation of *Acacia mangium* cuttings was observed to be weaker in May than in October (MONTEUUIS et al., 1995a). As a matter of fact, although slight under these latitudes, differences in photoperiod during the year have been noticed to have an effect on plant physiology as reflected by changes in vegetative characteristics, such as shoot growth variation and leaf fall observed locally for several tree species.

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Influence of different macronutrient solutions and growth regulators on micropropagation of juvenile *Acacia mangium* and *Paraserianthes falcataria* explants

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Abstract

The influence of five different macronutrient formulations and various growth regulators on micropropagation of single node explants from *Acacia mangium* and *Paraserianthes falcataria* seedlings was examined after 4 weeks and 8 weeks *in vitro*. The experiment was repeated 4 months later. On media lacking growth regulators, growth and development were significantly influenced by the different macronutrient solutions tested, although morphogenic responses could vary according to the species. For instance, *P. falcataria* displayed a greater ability for adventitious rooting than *A. mangium*. Overall, Knop macronutrient solution induced the weakest responses. Explant responsiveness was significantly influenced by the addition of 2.2 or 4.4 μM 6-benzyladenine combined with either 1.4 or 2.3 μM kinetin, 1.5 or 2.5 μM indolebutyric acid or 1.6 or 2.7 μM naphthaleneacetic acid. The various combinations of growth regulators tested were shown to inhibit the rooting ability of the explants, while stimulating the production of basal shoots for both species, and of axillary shoots only for *A. mangium*. In such experimental conditions, *A. mangium* displayed overall a greater potential for micropropagation than *P. falcataria*.

Abbreviations: BA – 6-benzyladenine; 3/4 B_5 – Gamborg et al. 3/4 strength medium; IBA – indole-3-butyric acid; 1/2 MS – Murashige and Skoog 1/2 strength medium: NAA – naphthaleneacetic acid; 2/3 QL – Quoirin and Lepoivre 2/3 strength medium; 2/3 SH – Schenk and Hildebrandt 2/3 strength medium

Introduction

Acacia mangium Willd. and *Paraserianthes falcataria* (L.) Nielsen, formerly known as *Albizzia falcataria*, are two tropical legume trees belonging to the Leguminosae family (Mimosoideae sub-family). They both originate from Papua New Guinea, and Eastern provinces of Indonesia, with extension to northern Australia (Queensland) for *A. mangium* (Gunn and Midgley, 1991), and more eastern to Samoa, Fiji and Hawaii for *P. falcataria* (Zabala, 1993).

Both exhibit impressive growth performances under the humid tropical conditions of South East Asia. They outperform many other fast-growing species such as *Eucalyptus deglupta* and *Gmelina arborea* on acid and poor soils. They can restore the fertility owing their natural nitrogen-fixing capacity (Sim, 1987). These remarkable growth performances have accounted for subsequent introductions of these two species in many tropical countries for pulpwood production for *A. mangium*, and for wider range of wood end-uses for *P. falcataria* (Zabala, 1993).

Despite this increasing crop potential for reafforestation, tree improvement programmes for *A. mangium* and more particularly for *P. falcataria* are still in their infancy. It is important to develop suitable tools to improve the genetic quality of the planting stock. In this regard, vegetative propagation is worth special attention, particularly tissue culture (Galiana et al., 1991; Mohd Basri and Alang, 1987; Rajadurai et al., 1987) as a possible alternative to overcome the limited success of more conventional techniques

faced for *A. mangium* (Darus, 1991; Monteuuis et al., 1995; Monteuuis, 1995; Poupard et al., 1994) and for *P. falcataria* (Chia, 1993; Zabala, 1993).

The purpose of this study was to assess the influence of different macronutrient formulations and growth regulator combinations on *in vitro* morphogenetic responses from juvenile explants of *A. mangium* and *P. falcataria*.

Materials and methods

Plant material and culture conditions

The influence of the macronutrients and the growth regulators on the *in vitro* morphogenic capacity of the explants was assessed separately and twice. The explants for both *A. mangium* and *P. falcataria* consisted of 1.5 cm long single node microcuttings, each with only one leaf and no shoot tip. For the first run of the experiment, they were excised from the epicotyl of 3-month-old *in vitro* germinated seedlings before being placed on the different culture media. Seeds of *A. mangium* and *P. falcataria* were soaked for 5 to 10 seconds and 1 min in hot water (90-95 °C) respectively, then surface disinfested by immersion in 70% ethanol plus 3 drops of Tween 80 for 10 min followed by 3 rinses in sterile ultra pure water before placement onto half-strength Murashige and Skoog (1962) macro and micronutrient solution supplemented with 50 mg l^{-1} myo-inositol, 500 mg l^{-1} casein hydrolysate, 2 mg l^{-1} glycine, 1 mg l^{-1} thiamine, 1 mg l^{-1} pyridoxine-HCl, 1 mg l^{-1} nicotinic acid, 20 g l^{-1} sucrose. This was referred to as the basal culture medium. For the second run of the experiment, the explants originated from the same source and were of the same type as those utilized for the first run, but were subcultured for 4 months with monthly transfer to the fresh basal medium before being exposed to the different treatments. At each transfer, the elongating explants were trimmed into 1.5 cm long single node microcuttings with only one leaf; the shoot tips were systematically discarded.

The influence of macronutrients on *in vitro* morphogenesis was assessed by placing the single node microcuttings on 5 different culture media totally lacking exogenous growth regulators, and varying only in their macronutrient composition. These consisted of "3/4 B_5" for 3/4 diluted Gamborg et al. (1968), "Knop" for Knop (1865), "1/2 MS" for half strength Murashige and Skoog (1962) , "2/3 QL" for 2/3 diluted Quoirin and Lepoivre (1977) and "2/3 SH" for 2/3 strength Schenk and Hildebrandt (1972). All these macronutrient formulations were supplemented with half-strength Murashige and Skoog (1962) micronutrients and the other components of the basal culture medium described above.

The growth regulator experiment consisted of placing the microcuttings on the basal 1/2 MS culture medium previously defined and supplemented with one of the following growth regulator combinations: (1) no growth regulator (control), (2) 2.2 μM BA + 1.4 μM Kinetin, (3) 2.2 μM BA + 1.5 μM IBA, (4) 2.2 μM BA + 1.6 μM NAA, (5) 4.4 μM BA + 2.3 μM Kinetin, (6) 4.4 μM BA + 2.5 μM IBA or (7) 4.4 μM BA + 2.7 μM NAA.

Explants were placed individually in 21 × 150 mm glass test tubes covered with polypropylene caps and containing 10 ml of specified culture medium solidified with 0.7% "high gel strength" Sigma agar. The pH of all the culture media was adjusted to 5.6-5.8 with KOH prior to autoclaving at 120 °C and 95 kPa for 20 min. After inoculation, the cultures were incubated under a 16-h photoperiod (50-60 μmol m^{-2} s^{-1}, "TLD 36W/84 Philips" fluorescent lamps) at 28/22 ± 2 °C light/dark.

Evaluation methods

The capacity for morphogenesis of the microcuttings was examined after 4 and 8 weeks of culture on the various media. The following traits were recorded for each of the 5 macronutrient formulations tested:

- percentage of microcuttings that produced axillary shoot(s) more than 1 mm long,
- length of the longest axillary shoot (more than 1 mm),
- number of pinnae per microcutting with a developed axillary shoot,
- percentage of rooted microcuttings,
- number of adventitious roots per rooted microcutting,
- number of secondary roots per rooted microcutting, and
- length of the longest secondary root per rooted microcutting.

The morphogenic criteria recorded to assess the influence of the growth regulators investigated were:

- the percentage of microcuttings that had produced axillary shoot(s) more than 1 mm long,
- the length of the developed axillary shoot more than 1 mm long,

- the number of basal shoots more than 1 mm long produced per microcutting,
- the length of the tallest basal shoot more than 1 mm long produced per microcutting,
- the percentage of rooted microcuttings,
- the percentage of microcuttings with a spongy basal callus.

Results are expressed as means based for both species on 96 explants, combining the first and the second runs of the experiment, each with 48 explants per experimental treatment. The influence of the main experimental factors (macroelements or growth regulators) on explant responsiveness, within each species and separately for data collected 4 weeks and 8 weeks after placement *in vitro*, was statistically analyzed using SPSS/PC+ statistical package (SPSS Inc., 1990). The data were subjected to the X^2-Pearson's test (proportions) or to a two-way factorial analysis of variance test (F test), with explant origin (1st or 2nd run of the experiment) as the second factor, after proper transformation as needed. The least significant difference (LSD) multiple comparison test was used to determine significant differences among the treatment means only when the F test was significant. A probability level of $p \leq 0.05$ was considered significant for all the statistical analyses.

Results and discussion

Influence of the macroelement formulations on explant morphogenesis

For *A. mangium* as well as for *P. falcataria*, the proportion of microcuttings that developed axillary shoot(s), the growth of these shoots and the number of pinnae produced varied significantly ($p < 0.001$) after 4 weeks and 8 weeks of culture according to the nature of the macronutrients (Table 1). Taller shoots were induced by 3/4 B_5 and 2/3 SH for *A. mangium* and by 1/2 MS for *P. falcataria*.

The macronutrients solutions tested had also a significant influence on the percentage of rooted microcuttings after 4 and 8 weeks for both species ($p < 0.001$), and on the number of adventitious roots produced after 4 weeks ($p = 0.023$) and 8 weeks ($p < 0.001$) only for *P. falcataria*. Overall, *A. mangium* explants rooted in lower proportions than *P. falcataria* ones, particularly after 8 weeks of culture, without any significant influence of the macronutrients except for Knop, and produced fewer adventitious roots, irrespective of the macroelements. For both species, the macrosalts had a marked effect after 4 and 8 weeks ($p < 0.001$, unless otherwise stated) on the number ($p < 0.002$ after 4 weeks for *A. mangium*) and on the length of the secondary roots formed per rooted explant. *A. mangium* rooted microcuttings produced longer secondary roots overall than *P. falcataria* explants.

Almost all the morphogenic traits observed for both species were significantly influenced by the different macrosalt formulations tested. The macroelements can actually influence the morphogenetic response of explants firstly through the total ionic concentration of the macrosalt solution, and secondly through the type of the macroelements provided. Emphasis was given in our study to this latter aspect by selecting five standard macrosalt formulations differing markedly from one another by the nature of the major elements included in the media. Preliminary experiments (data not shown) established that MS major elements diluted once (1/2 MS) gave better organogenic responses than MS full strength, as noticed also by Galiana et al. (1991) for shoot and root production in *A. mangium* explants. The strength of the different macrosalt solutions tested was therefore diluted in order that their total ionic concentrations ranged from 44.4 meq l^{-1} for the 2/3 SH solution to 50.4 meq l^{-1} for the 2/3 QL formulation, more adapted to micropropagation of tree species than richer solutions (George and Sherrington, 1984). Only Knop was maintained at its original ionic strength of 29.6 meq l^{-1}. Attempts at using a more concentrated Knop formulation to reach the total strength of the other media caused a liquefaction of the gelled medium. This lower total salinity can account for the weaker morphogenic responses observed overall on Knop, compared to the other solutions tested.

Influence of the growth regulator combinations on explant morphogenesis

The different treatments tested had a marked influence on the proportion of microcuttings which produced an axillary shoot and the length of it after 4 and 8 weeks ($p < 0.001$) for both species (Table 2). By comparison with the control, all the various growth regulators added to the culture medium stimulated axillary shoot formation in *A. mangium* explants. In contrast, the production of axillary shoots in *P. falcataria* was more likely to vary according to the different growth regulator combinations tested, particularly after 4 weeks in

Table 1. Mean values of the different morphogenic criteria examined for juvenile *in vitro* explants of *Acacia mangium* and *Paraserianthes falcataria* in relation to the various macronutrient formulations tested after 4 weeks and 8 weeks of culture.

Species	*Acacia mangium*					*Paraserianthes falcataria*				
Medium	3/4 B_5	Knop	1/2 MS	2/3 QL	2/3 SH	3/4 B_5	Knop	1/2 MS	2/3 QP	2/3 SH
% of explants with axillary shoot										
4 weeks	77.2bc	67.0c	84.3ab	92.9a	79.2bc	86.9ab	35.6d	91.4a	72.9c	75.6bc
8 weeks	75.8cd	68.1d	87.0ab	94.0a	83.2bc	97.6a	77.0b	100.0a	100.0a	98.6a
Axillary shoot length (mm)										
4 weeks	3.8ab	2.3d	3.5bc	3.1c	4.1a	5.2b	2.4b	6.8a	3.7b	3.9b
8 weeks	12.0a	6.6c	9.8b	9.2b	12.0a	17.8bc	8.8d	29.7a	19.6b	16.6c
No of pinnae per explant										
4 weeks	5.2ab	3.3d	4.8bc	4.4c	5.5a	3.1b	2.6b	4.4a	2.9b	2.8b
8 weeks	17.2ab	12.0d	15.8bc	14.2c	18.0a	12.5bc	7.4d	15.7a	14.0ab	12.4c
% of rooting										
4 weeks	67.4a	41.5b	69.6a	77.6a	68.8a	83.3a	35.6b	81.7a	71.8a	73.1a
8 weeks	71.4a	56.4b	77.4a	83.3a	75.8a	94.0ab	72.1c	90.1b	98.6a	97.1ab
No of adventitious roots										
4 weeks	1.0	1.0	1.0	1.0	1.0	3.4b	2.9b	4.3a	3.4b	3.4ab
8 weeks	1.3	1.2	1.4	1.3	1.4	7.0a	4.8b	7.7a	7.0a	7.5a
No of secondary roots										
4 weeks	13.7ab	9.1c	10.4c	10.9bc	15.0a	9.6b	2.5c	13.2a	5.9bc	7.8b
8 weeks	24.6a	20.0bc	16.3d	18.2cd	22.4ab	20.8a	9.0b	22.1a	19.4a	19.3a
Longest secondary root length (mm)										
4 weeks	18.4ab	9.1d	14.0c	17.4b	20.3a	11.5b	5.3c	14.9a	11.6b	13.3b
8 weeks	28.8a	22.4b	20.1b	27.7a	28.9a	18.6b	11.4c	21.6a	19.3b	18.4b

Means are based on 96 observations, corresponding to two repetitions of 48 explants per treatment at 4 month interval (see text for more information). Separately for each species, letters within each row distinguish means which are significanty different at the 5% level (X^2-Pearson's and LSD tests).

culture, which resulted all in an inhibition of axillary shoot elongation compared to the control.

For both species, the seven experimental treatments influenced significantly the mean number of basal shoots produced per microcutting after 4 and 8 weeks of culture ($p = 0.002$ for *P. falcataria* at 4 weeks, otherwise $p < 0.001$), and the length of the longest basal shoot ($p < 0.001$). In all situations, the production of basal shoots was stimulated by the growth regulators added to the culture medium. In particular, the combination of BA 4.4 μM + IBA 2.5 μM was very effective on *A. mangium* as compared to the control in which no shoot formation occurred. Overall, these basal shoots elongated better in *A. mangium*, especially when the microcuttings were exposed to BA + IBA, or BA + NAA, than in *P. falcataria* when placed in the same experimental conditions.

Overall, the combinations of growth regulators added to the culture medium resulted in a strong inhibition of the capacity of the microcuttings to form adventitious roots for *A. mangium*, and for *P. falcataria*, after 4 and 8 weeks of culture compared to the control ($p < 0.001$). The inhibitory effect was particularly strong for all the growth regulator treatments tested on *P. falcataria* whereas noticeable differences were observed among the same growth regulator treatments for *A. mangium*, especially after 8 weeks of exposure. BA combined to IBA or to NAA resulted in higher rooting rates than for the combination of BA and Kinetin.

Table 2. Mean values of the different morphogenic criteria examined for juvenile *in vitro* explants of *Acacia mangium* and *Paraserianthes falcataria* in relation to the various growth regulator treatments tested after 4 weeks and 8 weeks of culture.

Species	*Acacia mangium*							*Paraserianthes falcataria*						
Growth regulators (μM)	0	BA 2.2 + Kinetin 1.4	BA 2.2 + IBA 1.5	BA 2.2 + NAA 1.6	BA 4.4 + Kinetin 2.3	BA 4.4 + IBA 2.5	BA 4.4 + NAA 2.7	0	BA2.2 + Kinetin 1.4	BA 2.2 + BA 1.5	BA 2.2 + NAA 1.6	BA 4.4 + Kinetin 2.3	BA 4.4 + IBA 2.5	BA 4.4 + NAA 2.7
% of explants with axillary shoots														
4 weeks	84.3c	97.8ab	96.7 ab	95.8b	100a	100a	97.8ab	90.3a	91.5a	71.0bc	72.4bc	81.8ab	66.7c	67.7bc
8 weeks	87.0b	100a	96.4a	100a	100a	100a	100a	100a	97.0ab	81.1c	98.5a	98.4a	97.4ab	90.2bc
Axillary shoot length (mm)														
4 weeks	3.5ab	3.3b	3.6ab	3.6ab	2.6c	4.2a	3.8ab	6.9a	3.9bc	4.7b	2.5c	4.1bc	3.7cd	3.0d
8 weeks	9.8a	6.8cd	9.9ab	6.5cd	5.7d	9.0ab	8.2bc	29.7a	5.2b	6.0b	3.2c	5.2bc	4.5d	4.3cd
No of basal shoots														
4 weeks	0.0c	1.0b	1.2b	1.5b	1.1b	2.3a	2.6a	0.0d	1.1abc	1.6ab	1.2abc	1.1c	1.9a	1.3bc
8 weeks	0.0d	2.0c	2.1c	2.6bc	2.3bc	4.5a	3.7b	0.0d	1.7bc	1.9ab	1.4c	1.9ab	2.1a	1.7bc
Longest basal shoot length (mm)														
4 weeks	-	2.3c	3.3b	4.0a	2.6bc	5.1a	6.2a	-	2.1b	4.0a	2.2b	2.3a	1.7b	1.7b
8 weeks	-	5.1c	6.7b	8.5b	5.0c	10.0a	10.5a	-	2.8a	3.6ab	2.4c	2.5ab	2.4ab	2.0b
% of rooting														
4 weeks	69.0a	1.1c	6.7b	0.0c	0.0c	0.0c	0.0c	81.7a	0.0b	0.0b	0.0b	0.0b	1.2b	0.0b
8 weeks	77.4a	2.3cf	36.1b	4.4de	0.0f	14.7cd	25.9bc	90.1a	0.0b	0.0b	0.0b	0.0b	1.3b	0.0b
% of explants with basal callus														
4 weeks	0.0c	54.3b	92.2a	95.8a	68.1b	92.7a	97.8a	0.0d	38.0ab	30.1ab	44.2a	10.6c	39.3ab	24.6b
8 weeks	0.0c	62.8b	94.0a	100a	68.9b	93.3a	98.8a	0.0d	40.3bc	45.6abc	58.0a	33.3c	52.0ab	36.1bc

Means are based on 96 observations, corresponding to two repetitions of 48 explants per treatment at 4 month interval (see text for more information). Separately for each species, letters within each row distinguish means which are significantly different at the 5% level (X^2-Pearsons and LSD tests).

The production of a spongy callus at the base of the microcuttings was more pronounced for *A. mangium* than for *P. falcataria*. This response was significantly influenced after 4 and 8 weeks of culture by the different growth regulator combinations tested ($p < 0.001$). Except for the control, all induced callus formation with lower scores for BA + Kinetin combinations on *A. mangium*, and for BA 4.4 μM + Kinetin 2.3 μM on *P. falcataria*.

The plant growth regulators tested in this study were originally selected with a view to improving the capacity for micropropagation of *A. mangium* and *P. falcataria*, giving therefore more importance to shoot formation than to root formation. Previous observations established that *A. mangium* explants may respond well to cytokinins (usually 4.4 μM BA) by producing a large number of axillary buds (Darus, 1991; Galiana et al., 1991), but further development of these buds into elongated shoots to allow multiplication has so far been problematic. Information from the literature (Duhoux et al., 1986; Rao and Prasad, 1991; Semsuntud and Nitiwattanachai, 1991), and from preliminary experiments prompted us to opt for BA combined with Kinetin, IBA and NAA at the experimental concentrations tested, that corresponded to weight ratios of 2 and 1.6 suitable to promote new shoot elongation (George and Sherrington, 1984).

The predominance of the cytokinins may be responsible for the inhibition of adventitious root formation compared to the control, as previously reported (Galiana et al., 1991; Van Staden and Harty, 1988). This negative influence of cytokinins on adventitious rooting appeared to be particularly dominant for *P. falcataria* with a total inhibition of rooting ability, even when BA was associated with the auxins IBA or NAA, contrary to what was observed for *A. mangium*. Interestingly, the occurrence of basal callus formation tends to demonstrate that the tissues from the base of *A. mangium* microcuttings were overall more responsive to the various BA-auxin combinations tested than those of *P. falcataria*. This responsiveness concerned not only callus and root formation, but also the capacity for basal shoot production noticeably enhanced for *A. mangium* when exposed to 4.4 μM BA + 2.5

μM IBA or 2.7 μM NAA. From a practical point of view, the ability of the microcuttings to produce basal shoots has to be considered as the most realistic way to improve the multiplication rates, especially when using as explant single node microcutting from which only one single axillary shoot can be expected. This is particularly true for *P. falcataria* considering that for this species almost all the explants gave rise to axillary shoots in the absence of any growth regulator. However, histological investigations are still needed to determine the axillary or the adventitious origin of these basal shoots, which remains a basic question especially for genotypic stability.

The differences between data recorded after 4 and 8 weeks reflect the time needed for the explants to produce new visible morphogenetic features further to their exposure to the growth regulators. As shown, this could vary according to the morphogenic trait and the species concerned, and may provide information to determine the most suitable time for transferring the cultures onto a fresh medium, notwithstanding the influence of the constitution of the basal culture medium liable to change during time course (Minocha, 1987a,b).

Conclusion

The current study provides a lot of indications regarding the influence of different macroelement solutions and growth regulators on various morphogenic traits for *A. mangium* and *P. falcataria* microcuttings *in vitro*. Such information, lacking from the literature so far, especially for *P. falcataria*, can be used as guidelines to develop protocols more suitable for a certain type of morphogenesis *in vitro*, distinguishing basically between caulogenesis and rhizogenesis. For instance, the combination of 4.4 μM BA + 2.5 μM IBA seems to deserve particular attention for improving the multiplication rate whereas 2/3 QL can be recommended as a suitable macrosalt medium for testing different root promoting growth regulators. Further investigations along these lines are still needed aiming at developing ultimately efficient micropropagation protocols for superior genotypes of these two leguminous tree species of major economical importance.

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